Teresa Isabel de Amoreira Sousa e Silva Brás

MSc Engenharia Química e Bioquímica

Production of cynaropicrin extracts from *Cynara cardunculus* leaves and its use for development of wound dressing films

Dissertação para obtenção do Grau de Doutor em

Engenharia Química e Bioquímica

Orientador: Luísa Alexandra Graça Neves, Investigadora Auxiliar, Faculdade de Ciências e Tecnologia – Universidade Nova de Lisboa

Co-orientadores: João Paulo Serejo Goulão Crespo, Professor Catedrático, Faculdade de Ciências e Tecnologia – Universidade Nova de Lisboa

Maria de Fátima Pereira Duarte Ricardo, Investigadora Auxiliar, Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo

Júri:

Presidente: Prof. Doutor José Paulo Barbosa Mota

Arguente(s): Professor Doutor Armando Jorge Domingues Silvestre

Professor Doutor Frederico Castelo Alves Ferreira

Vogais: Professora Doutora Maria Manuela Estevez Pintado

Professora Doutora Isabel Maria Rôla Coelhoso

Professor Doutor Vítor Manuel Delgado Alves

Doutora Luísa Alexandra Graça Neves

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro de 2020

Production of cynaropicrin extracts from *Cynara cardunculus* leaves and its use for development of wound dressing films

“All our dreams can come
true, if we have the courage
to pursue them.”

Walt Disney

Acknowledgments

Aos longos dos anos de desenvolvimento deste trabalho, várias foram as aprendizagens a nível profissional e pessoal, que me tornaram mais rica em todos os aspetos. Nada disto teria sido possível sem a existência de Pessoas e Instituições que me apoiaram e às quais serei eternamente grata.

Quero começar por agradecer aos meus orientadores da FCT-UNL, Professor João Paulo Crespo e Doutora Luísa Neves, por me terem acolhido e decidido apoiar-me nesta caminhada. Ao Professor João Paulo, agradeço-lhe também toda a partilha de conhecimentos, a dedicação que teve, o espírito critico e os bons conselhos que me deu. À Doutora Luísa, um grande obrigada pela confiança de anos, que culminou com esta tese. Agradeço todo o apoio e por me ter permitido crescer tanto profissional como pessoalmente.

Agradeço à Doutora Fátima Duarte, do CEBAL, a sua enorme dedicação ao meu doutoramento, por me ter aceite como sua aluna, por me ter integrado no seu grupo de investigação. Um gigante obrigada pelo seu empenho, a sua dedicação, a sua sabedoria e a sua enorme paciência comigo. Agradeço por, em motivos de maior aflição não me ter deixado desmotivar, me ter feito ver o lado positivo e seguir em frente, tendo feito de mim uma profissional mais competente e uma pessoa mais completa emocionalmente.

À Professora Maria João Cabrita, do ICAAM, Universidade de Évora, agradeço a disponibilidade por me ter recebido no seu laboratório para a realização dos ensaios de Extração por Líquidos Pressurizados.

À Professora Adelaide Oliveira, da Escola Superior Agrária, do Instituto Politécnico de Beja, agradeço a disponibilidade demonstrada para utilização do equipamento de extração por Microondas.

Ao Professor Vítor Alves, do Instituto Superior de Agronomia, agradeço a disponibilidade para a realização dos ensaios de perfuração dos filmes. Agradeço também todo o apoio na interpretação dos dados obtidos e de toda a teoria inerente aos mesmos.

Ao Professor Armando Silvestre e à Doutora Patricia Ramos, agradeço a disponibilidade para a realização dos ensaios CG-MS e LC-MS e toda a partilha de conhecimentos relativa à caracterização química dos extratos analisados.

Agradeço à Dra Carla Brazinha, todo o apoio na avaliação económica. A paciência durante os telefonemas em tempos de pandemia, tendo a casa cheia de crianças, foi extraordinária.

À Professora Andreia Gomes, o meu obrigada por me ter recebido na Universidade do Minho, para a realização dos ensaios da IL-6. Obrigada pelo excecional acolhimento em Braga, por todo o auxílio com os ensaios e pela total disponibilidade para a discussão dos resultados.

Agradeço ao Doutor Jorge Mota Pereira e à Doutora Raquel Machado o terem cuidado do meu eu interior, da minha saúde mental, o interesse pelo meu trabalho, todas as palavras de incentivo e de confiança.

Um obrigada especial à Catarina Gonçalves da Universidade do Minho, pelo auxilio na realização dos ensaios da IL-6. Obrigada por teres abdicado dos teus fins de semana quando estive em Braga para que eu pudesse realizar em tempo record os ensaios.

Obrigada a todos no CEBAL, sem exceção. Cada um de vocês teve um papel importante durante estes anos, cada um à sua maneira. Um especial Obrigada a todos no Grupo de Compostos Bioativos, os que atualmente estão e os que estiveram durante esta aventura.

Maria Castro, obrigada por tudo! Obrigada por todos os conhecimentos transmitidos sobre cultura de células animais. Por me teres atendido o telefone quando tinha dúvidas inacabáveis. Mas, agradeço-te principalmente toda a amizade, todo o companheirismo e toda a fé que tiveste e tens em mim.

Ana Paulino, muito obrigada pelo teu carinho, amizade, confiança e palavras ternurentas para comigo, durante esta longa caminhada. Agradeço também todo o apoio no laboratório, sem dúvida importante.

À minha querida Daniela Rosa, agradeço a amizade, o carinho, as brincadeiras, as músicas animadas, o estar presente, o “estar lá”. Agradeço o teres aparecido de uma forma mais constante na minha vida, o nunca me teres deixado baixar a cabeça, me teres aturado as lamurias e a celebração das vitórias com a mesma euforia como se fossem tuas. Agradeço também, mas não menos importante, toda a ajuda ao nível da microbiologia e ao nível da recolha de amostras durante os ensaios de fracionamento.

À Ivone Torrado, agradeço a enorme amizade. Lado a lado comigo nesta caminhada de doutoramentos, tenho-te a agradecer os inúmeros conselhos, as inúmeras partilhas de possíveis soluções, a enorme criatividade. Agradeço por todo o apoio, dentro e fora do laboratório, e emocional. Agradeço por teres estado sempre lá, mesmo quando tudo foi difícil. Agradeço o teres apanhado as lágrimas, o teres motivado imensas gargalhadas, o teres celebrado as vitorias e ensinado a desvalorizar as derrotas. Obrigada pelo grande companheirismo.

À minha irmã, cunhado e sobrinhos, agradeço todo o apoio e compreensão por ter ido tão pouco ao Porto nesta caminhada.

Aos meus sogros, desejo agradecer o terem sido um pilar para os meus filhos, permitindo que eu muitas vezes estivesse ausente.

Aos meus pais, a educação e o amor que fizeram de mim a mulher que sou hoje. À minha mãe, agradeço toda a preocupação, todo o apoio dado, mesmo estando tão longe. Ao meu pai, presente no meu coração dia após dia, agradeço a herança genética da persistência, da resiliência e da superação. Tenho a certeza que estejas onde estiveres estás orgulhoso de mim.

Ao meu marido, Hugo e aos meus filhos, Inês e Diogo, agradeço-vos o facto de existirem na minha vida. Agradeço o fazerem de mim, uma mulher e mãe mais completa. Hugo agradeço-te todo o apoio, todas as questões pertinentes, toda a paciência, todo o ânimo, todo o amor, companheirismo. Inês e Diogo, a vocês agradeço a paciência que tiveram das muitas vezes que tive que dizer que precisava trabalhar e as vezes que estive ausente. Agradeço-vos o tornarem os meus dias cinzentos, em dias arco-íris. Agradeço cada beijo, cada abraço, cada ternura, cada carinho nada disto fazia sentido sem vocês na minha vida.

Abstract

Natural compounds derived from plants are presently an alternative for the discovery of new effective drugs, from which new products may be developed, potentially overcoming the current limitations of synthetic compounds for use in pharma and biomedical applications.

This thesis concerns the design and production of cynaropicrin enriched extracts from leaves of *Cynara cardunculus*, and their application in a chitosan matrix as wound dressing for anti-inflammatory drug delivery.

The design and production of cynaropicrin enriched extracts was performed considering the use of environmentally friendly extraction methodologies and biocompatible solvents. Selection of solvent and the extraction methodology took into consideration their effect upon cynaropicrin extraction yield, as well as energy expenditure and other operating costs. Among conventional and non-conventional extraction methodologies, ultrasound assisted extraction combined with ethanol presented the best results regarding the cynaropicrin extraction yield (55.00 mg/g Dry Weight) and energy expenditure (0.027 kWh/$g_{cynaropicrin}$).

Applied for the first time for cynaropicrin extraction from C. *cardunculus* leaves, ultrasound assisted extraction was firstly optimized regarding the pulse effect on cynaropicrin extraction yield. Comparatively to continuous mode, pulse mode (duty cycle of 25%) presented a reduction on energy consumption/$g_{cynaropicrin}$, as well as a better temperature control being selected and further optimized with a response surface methodology, using the Box-Behnken design model. The results obtained confirm the applicability of the response surface methodology for optimization of cynaropicrin extraction by pulsed ultrasound assisted extraction, from *C. cardunculus*.

Aiming the increase of the extract biological potential, fractionation of the optimized extract was performed by membrane processes. A Duramem® 200 membrane was selected for the retention of cynaropicrin while low molecular weight compounds, such as glucose and fructose, were removed by diananofiltration (after 5 diavolumes). As a result of the diananofiltration process, a global removal of 93.0% of glucose and 95.6% of fructose was obtained. An increase of the extract biological potential was achieved, when tested with a Bj5-ta human normal fibroblast cell line. The integrated process for production of cynaropicrin enriched extracts was economically assessed, leading to a pay-back period of 4.58 years, achieved for a production of 520 kg/ of extract.

Incorporation of the cynaropicrin enriched extract in chitosan films, was performed by the solvent evaporation method, with different concentrations of the extract. It was shown that the extract has a negative effect on the film tensile strength and on the fluid absorption capacity being these two effects strongly influenced by the amount of extract loaded in the chitosan films. With no cytotoxic effect on Bj5-ta cell line observed, for films with an extract concentration lower to 5%, a positive effect on skin inflammation was achieved. An 86% reduction on IL-6 cytokine levels production after exposure to chitosan with 5% extract, by indirect contact, was obtained being this effect positively correlated to the cynaropicrin content in the extract loaded into the chitosan films.

This work shows that cynaropicrin enriched extracts from *C. cardunculus* leaves, can be produced in a sustainable and environmentally friendly way, aiming the possibility to be scaled up for industrial applications. Besides that, its potential use as chronic skin wound dressing, allied to chitosan, is a promising approach overcoming the application of synthetic drugs, and/or potentiate their application spectrum on what regards to anti-inflammatory action.

Keywords: *Cynara cardunculus*; Cynaropicrin; Ultrasound assisted extraction; Organic solvent nanofiltration; Chitosan films; Anti-inflammatory activity

Resumo

Os compostos naturais derivados de plantas apresentam-se como uma alternativa no desenvolvimento de novos fármacos e produtos, com potencial para ultrapassar limitações relativas à utilização de compostos sintéticos para fins farmacêuticos e biomédicos.

O trabalho desenvolvido na presente tese focou-se na produção de extratos enriquecidos em cinaropicrina proveniente das folhas de *Cynara cardunculus*, e na sua incorporação em matrizes de quitosano, de forma a potenciar a resposta anti-inflamatória em feridas.

O desenvolvimento e a produção dos extratos enriquecidos com cinaropicrina foi efetuado considerando a utilização de metodologias de extração sustentáveis, bem como solventes biocompatíveis. A seleção do solvente e da metodologia de extração teve em consideração o rendimento de extração da cinaropicrina, assim como os custos energéticos e outros custos de operação. Entre as metodologias de extração testadas, os melhores resultados relativos ao rendimento de extração da cinaropicina (55,00 mg/g biomassa seca), e consumo energético (0,027 kWh/$g_{cinaropicrina}$) foram obtidos utilizando a extração assistida por ultrassons.

Pela primeira vez aplicada para a extração da cinaropicrina proveniente das folhas de *C. cardunculus*, a otimização da extração assistida por ultrassons foi inicialmente efetuada considerando o efeito pulsado no rendimento de extração. Comparativamente ao modo contínuo, o modo pulsado (ciclo de trabalho de 25%) apresentou uma redução no consumo energético/$g_{cinaropicrina}$, assim como um melhor controlo de temperatura, sendo selecionado e posteriormente otimizado por metodologia de superfície de resposta, usando como modelo o planeamento Box-Behnken. Os resultados obtidos confirmam a aplicabilidade da metodologia de superfície de resposta na otimização da extração assistida por ultrassons pulsado da cinaropicrina a partir das folhas de *C. cardunculus*.

Com o objetivo de potenciar a resposta biológica realizou-se o fracionamento do extrato otimizado, por processos de separação com membranas. A membrana Duramem® 200 foi selecionada, pela sua capacidade de retenção da cinaropicrina, enquanto compostos com baixo peso molecular, como a glucose e a frutose, foram removidos por diananofiltração. Como resultado do processo de diananofiltração foi possível obter uma remoção global de 93,0% de glucose e 95,6% de frutose. O extrato fracionado foi testado na linha celular de fibroblastos humanos, Bj5-ta, verificando-se um aumento do seu potencial biológico, comparativamente com o extrato inicial. De acordo com a avaliação económica do processo integrado, o tempo de retorno do investimento é de 4,58 anos para uma produção anual de 520 kg de extrato seco.

A incorporação de extratos enriquecidos em cinaropicrina, em filmes de quitosano foi realizada através do método de evaporação de solvente, com diferentes concentrações de extrato. O extrato apresenta um efeito negativo na resistência à tração dos filmes, assim como na sua capacidade de absorção de fluidos, sendo esses efeitos fortemente influenciados pela quantidade de extrato incorporado nos filmes de quitosano. Sem efeito citotóxico na linha celular Bj5-ta, para filmes com

uma concentração de extrato inferior ou igual a 5%, foi obtida uma redução de 86% nos níveis de produção da interleucina-6, após exposição ao extrato de filme. A resposta anti-inflamatória obtida encontra-se positivamente correlacionada com a concentração de cinaropicrina, no extrato incorporado nos filmes de quitosano.

O trabalho desenvolvido demonstra a possibilidade de produção sustentável de extratos enriquecidos em cinaropicrina a partir das folhas da *C. cardunculus*, evidenciando o potencial para aplicação industrial. Os extratos obtidos, quando incorporados em matrizes de quitosano, aumentam a ação anti-inflamatória, evidenciando a sua relevância para a aplicação e simultaneamente ultrapassando limitações inerentes da aplicação de fármacos sintéticos.

Palavras chave: *Cynara cardunculus*, Cinaropicrina, Extração assistida por ultrassons; Nanofiltração de solventes orgânicos; Filmes de quitosano; Atividade anti-inflamatória.

Abbreviations

ANOVA	Analysis of Variance
AO	Acridine Orange
ATCC	American Type Culture Collection
BE	Batch Extraction
BSA	Bovine Serum Albumin
CUAE	Continuous Ultrasound Assisted Extraction
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
DW	Dried Weight
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
EtOH	Ethanol
EtPUAE	Ethanolic Pulsed Ultrasound Assisted Extract
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FI	Flow-rate Indicators
FT-IR	Fourier-Transform Infrared Spectroscopy
HPLC	High Pressure Liquid Cromatography
HSP	Hansen Solubility Parameters
HSPiP	Hansen Solubility Parameters in Practice
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-1β	Interleukin 1β
LPS	Lipopolysaccharides from Escherichia coli O111:B4
MAE	Microwave Assisted Extraction
MeOH	Methanol
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
MW	Molecular Weight
MWCO	Molecular Weight Cut-Off
n	Number of variables
OSN	Organic Solvent Nanofiltration
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PI	Pressure Indicators
PI	Propidium Iodide
PLE	Pressurized Liquid Extraction
PUAE	Pulsed Ultrasound Assisted Extraction
R-Squared	Coefficient of determination

RSM	Response Surface Methodology
SEM	Scanning Electron Microscopy
SL	Sesquiterpene Lactones
SMILES	Simplified Molecular Input Line Entry Syntax
SRNF	Solvent Resistant Nanofiltration
TGA	Thermogravimetric Analyses
TGFα	Transforming Growth Factor - α
TGF-β	Transforming Growth Factor - β
TNF	Tumor Necrosis Factor
TPC	Total Phenolic Content
TRITC	Tetramethylrhodamine Isothiocyanate
UAE	Ultrasound Assisted Extraction
VEGF	Vascular Endothelial Growth Factor
Y-MB	Yamamoto-Molecular Break

Variables

A	Membrane area, m^2
A, B and C	Response surface methodology variables
b_{ii}	Quadratic effect coefficient
b_{ij}	Interactive effect coefficient
C.V.	Coefficient of variation, %
$C_{i,f}$	Concentration of solute i in feed, mol/m^3
$C_{i,p}$	Concentration of solute i in the permeate, mol/m^3
C_p	Heating capacity of the mixture (solvent + sample), J/g.K
D	Diavolumes
dT/dt	Rate of temperature rise, K/min
E	Microwave energy absorbed, J/min
IC_{50}	Half-maximal inhibitory concentration, µg/mL
J_v	Permeate volumetric flux, $L/(m^2.h)$
Lp	Membrane permeability, $L/(m^2.h.bar)$
Lp_i	Solvent or extract i permeability, $L/(m^2.h.bar)$
RED	Relative Energy Difference
R_i	Compound i apparent rejection, %
S/L	solid/liquid ratio, g/mL
SD	Membrane swelling degree, %
t	Diafiltration time, h
tan β	Dissipation factor
t_{OFF}	Time of pulse interval on OFF mode, s
t_{ON}	Time of pulse interval on ON mode, s
t_{total}	Number of pulse repetitions (t_{ON} + t_{OFF})
V	Volume, mL
V_D	Predicted diavolumes
VEtOH	Ethanol volume added during diananofiltration, L
V_f	Feed volume in the beginning of the experiment, m^3
Vr	Retentate volume, m^3
VRF	Volume Reduction Factor
w	Weight, g
WD	Weight of the dried membrane, g
$W_{loss,\ i}$	Compound i weight loss, %
W_S	Weight of the membrane after 24 h of contact with EtPUAE, g
Y_i	Response surface methodology response

Greek letters

δ_D	Dispersion forces
δ_H	Hydrogen bonding
δ_P	Permanent dipole forces
ΔP	Transmembrane pressure, bar
$\Delta \pi$	Osmotic pressure difference, bar
ε''	Dielectric loss factor
ε'	Dielectric constant (F/m)

Contents

LIST OF FIGURES

LIST OF TABLES

1

Background and Motivation

1.1. Production of bioactive extracts

Presenting a huge diversity of chemical substances, natural products have for long been recognized as an important source of therapeutically effective medicines. However, in the last decades, the pharmaceutical industry has been focused on libraries of synthetic compounds, as a drug discovery source, using high throughput screening platforms. The structural diversity of natural compounds far exceeds the capabilities of synthetic organic chemists, within the laboratory facilities, for new medicines development. In the last decades, a declining trend in the number of new drugs reaching the market has been observed, raising renewed scientific interest in drug discovery from natural sources for use against cancer, microbial infection, inflammation and other diseases [1-4].

The plant kingdom represents a magnificent, valuable and diverse source of natural chemical compounds with an estimation range between 200 000 to 1 000 000 different metabolites [5]. With an important role in plant protection from biotic and abiotic stress, secondary metabolites present a great diversity, derived from the infinite combination of fundamental functional groups, originating compounds with peculiar chemical and physical characteristics (e.g. solubility, melting point and reactivity) [6]. Frequently referred as bioactive compounds, secondary metabolites possess the ability to interact with living tissues, promoting pharmacological or toxicological effects in humans and animals and therefore, presenting therapeutic potential [4].

The extraction of bioactive compounds involves separation of target compounds from the plant matrix in a nearly pure form [7, 8]. Extraction methodologies must be thought carefully to ensure a successful isolation of the compounds of interest, as well as preserve their biological activity [9]. Besides that, the structural diversity and physicochemical properties of bioactive compounds are two of the factors that determine which extraction technique should be used [10]. Non optimized extraction methods are often time and energy consuming, induce the use of large quantities of

solvents, being these in their majority potentially toxic, harmful for the environment and users, generating large amounts of wastes [11].

Worldwide, the food processing sector and the chemistry industry are both related with the extraction of bioactive compounds from plants, and represent, respectively, 16% and 26% of the most energy-intensive industries [11]. Largely applied, conventional extraction processes (maceration, infusion, decoction and boiling under reflux) are quite laborious, time- and energy-consuming, involve large amounts of solvents (e.g. hydrocarbons, alcohols and chloroalkanes) and, ultimately, may cause degradation of target molecules and partial loss of volatiles [12]. Despite the high energy consumption and the large amount of solvents involved, the extraction yield is often very low [13]. Therefore, in the past decades with the increasing concern of environmental and safety conditions, safer and more efficient extraction techniques have been considered, based on a reasonable compromise between economic, social and environmental requirements [14]. These techniques include microwave-assisted extraction (MAE), ultrasound assisted extraction (UAE), supercritical fluid extraction (SFE), and pressurized liquid extraction (PLE) [8]. These new methods typically require less time, lower solvent consumption and lower energy input, leading to increased extraction yield, and also presenting easiness for upscaling and automation [9]. Among the key considerations related to an extraction process, the selection of appropriate solvents for bioactive compounds extraction from plants should firstly be considered. Most important, the basis for solvent selection depends on the specific nature of the bioactive compound(s) to be isolated [15].

From the secondary metabolites that can be found in plants, sesquiterpene lactones (SL) are one of the most prevalent and biologically significant group, comprising over 5 000 known compounds [16]. SL can be generally included in Cactaceae, Solanaceae, Araceae and Euphorbiaceae families, with a high prevalence in the Asteraceae, where they can be found all-over [17].

With an interesting biological potential, the scientific interest on SL has exponentially increased in the last years (Figure 1.1). However, very few studies have been centered on SL extraction and its optimization, being this subject, described in detail in Chapter 2 of this thesis. Several authors have demonstrated the SL potential for biomedical applications due to their anti-tumoral, antimicrobial, antimalarial and anti-inflammatory potential [18-26], among others.

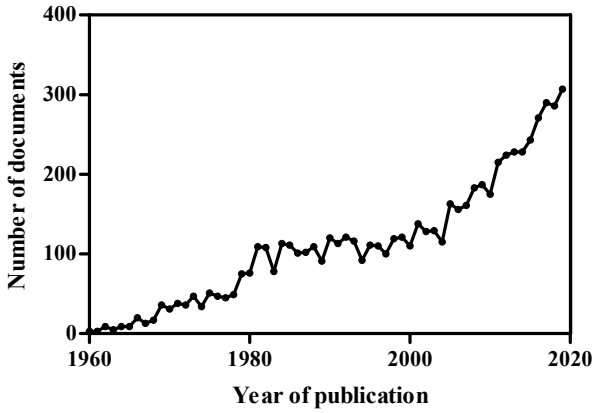


Figure 1.1. Number of peer reviewed published documents, per year (1960-2019), regarding the theme sesquiterpene lactones (Scopus accessed in 31th May 2020).

With an estimated biomass production that can range from 7.8 – 20.0 ton DW/ha, *Cynara cardunculus* (Asteraceae family) (Figure 1.2) presents a high potential from the perspective of a complete plant exploitation, being a source of: lignocellulosic compounds (stems and whole plant); oil (seeds); cardosins (flower), with a high use in the cheese industry and as a source of diverse bioactive compounds (heads, leaves and roots), with leaves representing from 33.1% till 48.4% of the total biomass average weight [27, 28].

Among the bioactive compounds that can be extracted from *C. cardunculus* leaves, SL are the most prevalent group detected (≈94.5 g/kg DW), mostly represented by cynaropicrin (≈87.4 g/kg DW) [29]. Cynaropicrin (chemical structure presented in Figure 1.3) has shown a high biological potential and has demonstrated extraordinary pharmacologic properties such as anti-hyperlipidemic [30], anti-trypanosomal [31], antifeedant [32], antispasmodic [33], anti-photoaging [34], antiproliferative [18] and anti-inflammatory response [25, 35]. Not restricted to the *Cynara* genus, it is in *C. cardunculus* leaves that cynaropicrin is largely available and, although its biological potential is already known [18, 25, 26, 31, 33-37], minor efforts have been done in order to increase its content in *C. cardunculus* leaves extracts, for final pharmacological or food industry.

It is therefore challenging to consider the cynaropicrin extraction from *C. cardunculus* leaves, following a concept of environment-friendly extraction. In addition to considering also the mentioned drawbacks of conventional methodologies applied for SL extraction *versus* the potential of non-conventional ones. Aiming this, Chapter 3 discusses the influence of the extraction solvent and methodology on the recovery of cynaropicrin from *C. cardunculus* leaves.

Figure 1.2. *Cynara cardunculus*.plant with detail of flower and leaves

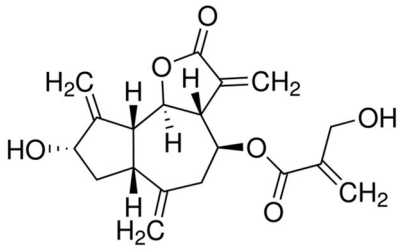


Figure 1.3. Cynaropicrin chemical structure.

Plant extracts may include compounds and/or fractions with distinct biological potential. Advances in the recognition of the referred potential led to the development of strategies for the fractionation and/or isolation of natural compounds from plant extracts being chromatographic processes the most applied (at lab scale) [38]. Besides that, due to solvent toxicity and/or low activity, extracts are typically converted into powder or concentrated oils to become commercially available products [39, 40]. Traditional approaches used for concentration of biological active compounds include simple steam and vacuum distillation almost always requiring high temperatures or high energy input, which turns them unappropriated [36, 41-43].

In recent years, more environmental friendly techniques have been investigated and used for the separation, purification and concentration of bioactive compounds, promoting a reduction of processing time and solvent consumption, as well as an increase of the final recovery yield [44]. Recently, membrane separation technologies have been recognized as powerful tools for the purification and concentration of various solutions (e.g., juices, extracts, whey), as well as the fractionation of valuable compounds from by-products of the agro-food industry [45]. Membrane processing requires mild operating temperatures with no phase change, avoiding thermal degradation, with preservation of functional compounds. Moreover, chemical additives are not

required, membrane separation technologies are modular and easy to scale-up, and may present high selectivity for the target compounds if adequate operating conditions are used. Membrane processing may, therefore, enable a more rational utilization of raw materials and recovery and reuse of by-products. In addition, membrane technologies respond efficiently to the requirements of so-called “process intensification”, allowing drastic improvements in manufacturing and processing, substantially decreasing the equipment-size/production-capacity ratio, energy consumption, and waste production [46, 47]. These potential advantages associated with low energy requirements, usually define membrane technologies as a sustainable processes [48-51].

However, the solvents applied for extraction of bioactive compounds, namely SL, are mostly organic. Although solvent resistant nanofiltration (SRNF) has been applied with success in the pharmaceutical industry, such as in the removal of genotoxins from active pharmaceutical ingredients (APIs) [52], purification of APIs [53], catalyst recycling [54], and solvent recycling and exchange [55], its utilization is still scarce mainly due to the lack of SRNF commercially available membranes. Recent developments of organic stable nanofiltration membranes, with tuned molecular weight cut-off (MWCO), has broadened SRNF opportunities for molecular separation in this field [56], evolving fast and opening new perspectives in pharma and agro-food industries.

Following the topic of extraction optimization, Chapter 4 addresses the study and process development to obtain a cynaropicrin rich fraction using membrane technologies. In addition, an integrated process including extraction and fractionation will be presented, as well as its and economic feasibility.

1.2. Bioactive dressings for transdermal drug delivery

Skin, the largest body organ, has as main function to serve as a barrier to harmful environments preventing pathogens to enter the body [57]. Wound healing is a dynamic, complex and systematic process of tissue regeneration and growth, traditionally explained in terms of four major stages (Figure 1.4): i) coagulation and haemostasis phase (immediately after injury); ii) inflammation (shortly after injury), during which swelling takes place; iii) proliferation, where granulation tissues form and wound begins to contract, and iv) maturation, in which remodeling of new tissues takes place [58-60]. The proper occurrence of these stages requires the presence of growth factors and cytokines. However, in some cases, these factors are not sufficiently present, or are significantly upregulated and may delay the healing process from its normal cascade or completely halt it (e.g. chronic wounds) [61].

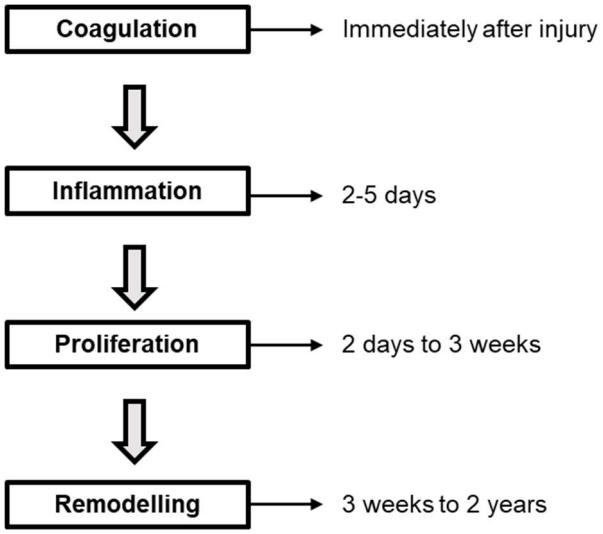


Figure 1.4. A brief view of the wound healing cascade (adapted from [62]).

Extensive inflammation plays a major role in the disruption of the normal healing cascade presenting a significant psychological, physical and financial burden for patients [63].

Compared to systemic delivery, localized controlled release systems reduce undesired side effects, such as toxicity and suboptimal delivery and provide spatio-temporal control over the drug dosage directly at the wound site. Besides that, it protects the drug from metabolic deactivation, and maintains the drug concentration at a desired level over a prolonged period of time [61].

Besides drug delivery, appropriate and immediate coverage of the wound area with an adequate dressing is essential for wound protection in order to accelerate wound healing. Their primary function is to keep the wound dry, allowing evaporation of wound exudates and preventing the entry of harmful bacteria into the wound. In recent years, wounds treatment has been revolutionized based on a better understanding of the underlying molecular and cellular abnormalities that prevent wound healing [62, 64].

In a constant need to improve patient life quality and decrease ambulatory costs, efforts have been made, in the last two decades, towards the design of new materials for wound dressing, considering the different phases involved. Current research has shown benefits associated with the incorporation of bioactive materials on opposition to those that are inert, due to their biocompatibility, biodegradability, non-toxicity, as well as ability to interact with the biological environment and to influence cellular functions as proliferation, or histoarchitectural tissue organization [65]. Examples of bioactive materials are hydrocolloids, alginates, collagens, chitosan, chitin, and chitin derivatives [66-71].

Chitosan, a biopolymer produced by deacetylation of chitin is mainly obtained from crustacean shells, has been regarded as one of the most promising materials for wound dressing applications [72]. It is a copolymer that consists of β - (1→4)-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose units (Figure 1.5). Manufacture of edible films or food packaging materials based on chitosan has been widely studied [73-75]. Additionally, due to its unique properties such as biodegradability, non-toxicity, anti-bacterial effect, and biocompatibility it also

gained interest in the biomedical research area. In fact, chitosan's monomeric unit, N-acetylglucosamine, occurs in hyaluronic acid, an extracellular macromolecule that is important and widely used for wound repair [76].

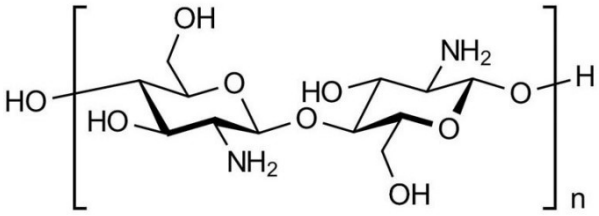


Figure 1.5. Chitosan chemical structure.

Within the past 20 years, a considerable amount of studies have been published on chitosan and its potential use for drug delivery systems [77, 78]. On opposite to all other polysaccharides, chitosan has a cationic character because of its primary amino groups, responsible for properties such as controlled drug release, mucoadhesion, *in situ* gelation, transfection, permeation enhancement, and efflux pump inhibitory properties [79]. Although the use of chitosan for drug delivery systems has been highly studied, specific applications of chitosan in wound dressing with anti-inflammatory properties is still scarce and challenging. Loading with natural based anti-inflammatory compounds such as cynaropicrin into chitosan films, allying chitosan and *C. cardunculus* leaves extract, represents a high opportunity for chronic wound healing.

Based on this, in Chapter 5 of this thesis, chitosan films are used as polymeric matrices for incorporation of *C. cardunculus* leaves' extracts, aiming the development of skin drug delivery biomaterials.

The overall objective of this thesis is the study and optimization of methodologies for production of bioactive extracts, fractionation of cynaropicrin as the target compound, and its incorporation in a chitosan matrix for anti-inflammatory drug delivery for use in chronic skin wounds. For extract production, *C. cardunculus* leaves are used as plant matrix due to their high content in the SL cynaropicrin. Optimization of extract production is followed by fractionation using sustainable technologies (membrane processing). Finally, the development of chitosan-based films comprising the bioactive target compound for chronic skin wounds and, therefore, the development of a biocompatible wound dressing loaded with the anti-inflammatory cynaropicrin-rich extract, without loss of physical and biological chitosan properties, will be assessed.

1.3. Research strategy

Considering the global objective previously mentioned, the work developed in this thesis is organized in two major sections:

a) Optimization of cynaropicrin extraction methodologies from *C. cardunculus* leaves and extract fractionation by membrane processing;

b) Development of chitosan-based films loaded with cynaropicrin and assessment of their biological anti-inflammatory properties.

A scheme considering the different tasks performed in this PhD is presented in Figure 1.6.

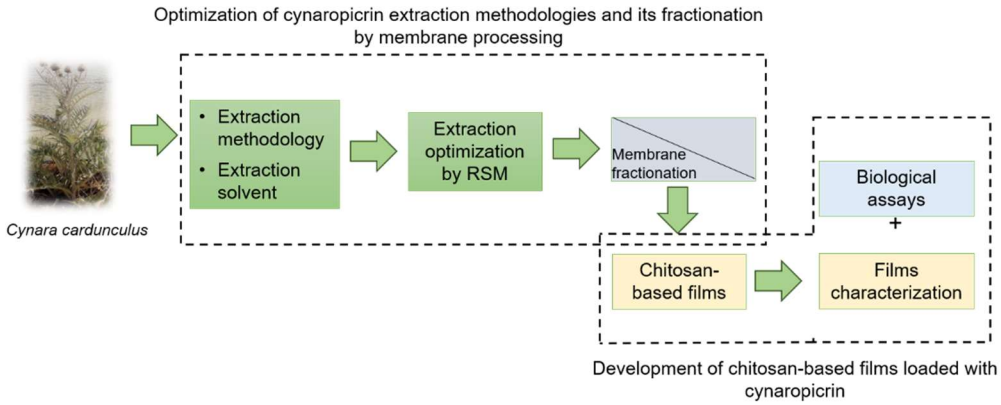


Figure 1.6. PhD tasks scheme. RSM: Response Surface Methodology

According to the previously mentioned, in section 1.1, and considering the lack of information regarding the use of different extraction solvents and methodologies, as well as their effect upon SL extraction yield, cynaropicrin extracts were optimized using selected solvents and diverse extraction methodologies. For that, conventional and non-conventional extraction methodologies were selected and compared with Batch Extraction (BE), commonly regarded as a reference extraction methodology. For non-conventional extraction methodologies, Ultrasound Assisted Extraction (UAE), Microwave Assisted Extraction (MAE) and Pressurized Liquid Extraction (PLE) were chosen. To study the solvent extraction effect, different sustainable solvents, with distinct chemical affinity towards cynaropicrin were selected. As a control extraction solvent and procedure, dichloromethane and soxhlet extraction were selected, according to the results previously published for cynaropicrin extraction from *C. cardunculus* leaves [29].

After identification of the best solvent and methodology for cynaropicrin extraction from *C. cardunculus* leaves, the question that arose was: how can the cynaropicrin extraction yield and extract content be optimized? What extraction intrinsic parameters influence the cynaropicrin extraction yield and extract content? To answer these questions, optimization by Response Surface Methodology (RSM) was performed. Application of RSM for optimization of the extraction procedure presents advantages compared to classical one-variable-a-time optimization, such as the generation of large amounts of information from a reduced number of experiments and the possibility of evaluating the interaction effect between different variables and their impact on the response [80]. Among the experimental design methodologies available, the Box-Behnken design was selected, considering its efficiency and economy when compared to other 3^k design methodologies, mainly when a large number of variables are involved (Figure 1.7).

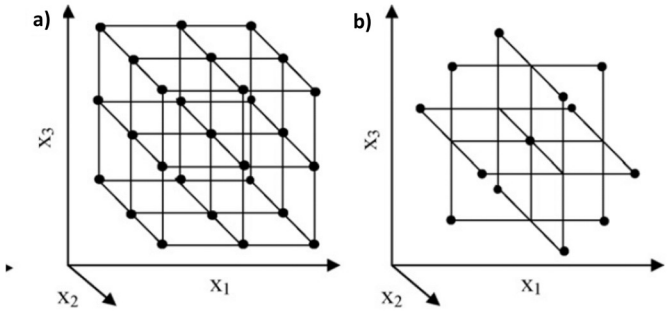


Figure 1.7. Experimental design based on three variables and three levels: a) factorial design; b) Box-Behnken design (adapted from [80]).

After optimization of extract production, the extracts with the highest biological potential were fractionated by membrane processing in order to remove non-desired compounds. Hydrophilic nanofiltration membranes, NF270 and NF90, and an organic solvent resistant nanofiltration membrane, Duramem 200, were selected for this purpose, considering the alcoholic character of the extract.

The overall process of extraction, fractionation and drying of the final extract was evaluated from a technical and economical perspective, taking also into consideration the need for permeate reuse, to minimize the environmental and economic impact of the entire process.

Finally, for the development of chitosan-based films loaded with *C. cardunculus* leaves extracts, a solvent evaporation method was used. In order to understand the effectiveness of extract incorporation, as well as its effect on the chemical and mechanical properties of the produced films, different characterization techniques were used. A chitosan film without extract incorporation was also prepared for comparison purposes.

Considering the extract delivery performance, cytotoxicity assays were performed with human skin fibroblasts as well as drug release *in vitro*. For the anti-inflammatory biological potential, IL-6 interleukin was selected, due to its proved influence on delayed wound healing [81].

This thesis presents a comprehensive approach for the production of SL bioactive extracts using *C. cardunculus* leaves as biomass source, with cynaropicrin being the target bioactive compound. A sustainable strategy is presented to reduce the environmental impact associated with production, assuring simultaneously the technical and economic feasibility of the process.

1.4. Thesis outline

This thesis comprises the work developed during this PhD project and is organized in seven chapters. The first chapter introduces the thesis background and motivation as well as the research strategy followed. This introductory chapter is followed by a literature review regarding extraction methodologies and solvents, used for SL recovery. The next four chapters are original scientific papers, accepted or under review, that describe the experimental work performed as well as the main results obtained. The thesis closes with a chapter that summarizes the overall conclusions of the work and proposes future research lines in this field.

The work developed in this thesis resulted in five scientific papers, either accepted, under review or in preparation to be submitted. Additionally, it also led to the grant of an European Patent Request, presented on the annex section of this thesis.

A short description of each chapter is presented:

Chapter 1 introduces the thesis background and motivation, and the research strategy followed.

Chapter 2 reports the state-of-the-art regarding SL solvent extraction and most used methodologies, focusing on the literature published during the last ten years.

Chapter 3 comprises a study of different solvents and methodologies for extraction of cynaropicrin from *C. cardunculus* leaves and their impact on the extraction yield. In this study, non-conventional extraction methodologies are compared among them and with conventional ones. Besides the impact in terms of extraction yield, the energy consumption in each process was also assessed. The extraction solvent selection was restricted to biocompatible solvents using Hansen solubility parameters, applying the Hansen Solubility in Practice (HSiP) software, to anticipate the SL extraction potential of different extraction solvents.

Chapter 4 describes the extraction methodology optimization process. This approach was applied for optimization of ultrasound assisted extraction, considering two steps. Firstly, the pulse effect on cynaropicrin extraction was studied, followed by optimization of extraction parameters using the response surface methodology with Box-Behnken. Optimization was performed for the cynaropicrin extraction yield and for the cynaropicrin concentration in the extract.

Chapter 5 describes how an optimized extract can be fractionated by diananofiltration, followed by concentration by nanofiltration. In this study different hydrophilic and organic solvent resistant nanofiltration membranes were tested, aiming the removal of compounds (sugars) with low biological activity, assuring simultaneously a maximum cynaropicrin recovery. Fractionated and non-fractionated extracts were compared in terms of their cytotoxicity in vitro, using human skin fibroblasts, Bj5-ta cell line.

Chapter 6 discusses the development of chitosan-based films loaded with C. cardunculus extracts. In this study, different extracts were loaded in chitosan films and their chemical, physical and biological characterization was carried out.

Chapter 7 presents the overall conclusions of the PhD project and suggests future lines of research.

2

The role of solvent and extraction methodology upon sesquiterpene lactones extraction: overview of the last ten years of research

Brás, T., Neves, L. A., Crespo, J. G., & Duarte, M. F. (2020). The role of solvent and extraction methodology upon sesquiterpene lactones extraction: overview of the last ten years of research. Phytochemistry Reviews, in preparation to be submitted

2.1. Abstract

Sesquiterpene lactones (SL), one of the major groups of secondary metabolites from plants, comprises more than 5000 compounds, with high biological potential. Due to their chemical structure, SL synthesis is hard, implying high energy and environmental costs. Access to SL biological potential could be achieved by solid-liquid extraction, being solvent and extraction methodology key factors for extraction to succeed. During the last decade, SL research was mainly focused on compounds identification, with a large range of solvents being applied, as well as the use of different conventional extraction methodologies. However, there is still a lack of scientific knowledge, regarding the extraction solvent and methodology effect upon SL extraction from plants, and its resulting bioactivity.

With this review, a perspective of extraction solvents and methodology impact on SL extraction efficacy is given, highlighting pertinent aspects and opportunities that can be applied for future research regarding SL extraction for economic valorization.

Keywords: Sesquiterpene lactones; extraction methodology; extraction solvent; *Cynara cardunculus*; cynaropicrin

2.2. Introduction

Plants have been used for centuries, as sources of remedies, used in different medicinal approaches due to the high structural chemical diversity, combined with remarkable biological activities. Within the last years, the interest for natural products, has led to the development of novel therapeutic agents, promoted an emerging interest on different plant derived natural compounds, such as sesquiterpene lactones (SL). With a high prevalence on the Asteraceae family, comprising over 5000 known compounds, SL are one of the most prevalent and biologically significant group of secondary metabolites found across the plant kingdom [16, 17]. Although with a high prevalence, SL ecological role is not yet clearly defined, with some studies characterizing SL by been deterrents to herbivores, as well as presenting toxic properties against pathogens, such as bacteria and fungi [17, 82, 83]. Nevertheless, other studies demonstrate that SL actions can either be toxic to insects while attracting predators, or as a "warning to other nearby plants, allowing them to prime their defenses systems [84].

Characterized for being lipophilic colorless constituents, bitter and relative stable, many scientific studies report that leaf capitate glandular trichomes (CGTs) are the main site of SL biosynthesis in Asteraceae family, where they are mainly identified, although some studies detected SL presence in root exudates [85-96]. With a chemical structure, comprising a fifteen carbon atoms skeleton, SL can be organized by subtypes according to the arrangement of their core skeleton (Figure 2.1), with germacranolides, eudesmanolides and guaianolides, as the most representative subtypes [84].

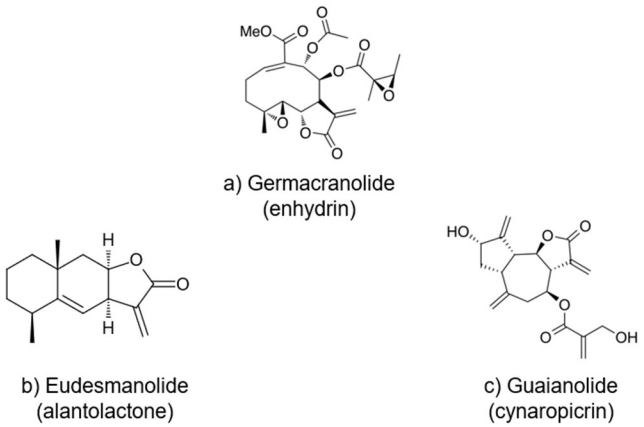


Figure 2.1. Chemical structure of a) germacranolide, b) eudesmanolide and c) guaianolide sesquiterpene lactone types with enhydrin, alantolactone and cynaropicrin as respective examples.

SL research interest was from the very beginning directed by its biological potential and goes back to 1936, where helenanin from *Helenium autumnale* leaves, due to its insecticide potential, was studied and extracted by percolation with chloroform [97]. On opposite to other plant-based natural compounds, scientific interest on SL has slowly increased during the last century. However, in the last ten years there was an exponential interest mainly driven by its biological potential, with anti-inflammatory, anti-tumoral, antiviral, antibacterial or cytotoxicity effects as examples. With more than 180 scientific peer reviewed papers published between 2010-2019, concerning the subject of

sesquiterpene lactones, *Artemisia* [98-114], *Cynara* [18, 29, 34, 37, 115-122], *Inula* [92, 94, 96, 123-131] *Tanacetum* [93, 132-135] and *Vernonia* [136-142] were the main species studied.

Due to its complex chemical structure, natural compounds synthesis is hard and implies high energy and environmental costs. Moreover, several studies have demonstrated that synthetic molecules not always carry the same broad range of pharmacoactivities, and simultaneous have lower bioavailability and tolerability, which may induce many side effects on human health [143]. Therefore, it is important to find new efficient, and cost-effective extraction methods that constitute an alternative to SL synthesis, in order to benefit SL biological potential. Besides that, the influence of extraction procedures and extraction parameters upon SL extraction from plants, is still shortly explored.

This review intends to document and summarizes the solvents and extraction methodologies applied during the last decade for SL extraction, unveiling trends and guidelines for a more effective and sustainable approach. The specific extraction of cynaropicrin is presented at the end of this review, as a case study.

2.3. Extraction solvents

Responsible for the penetration on plant matrix and for compounds solubilization, the success of solid-liquid extraction of bioactive compounds from plants largely depends on an appropriate solvent selection. The proper solvent may lead to higher extraction yield, as well as final extract purity and eventually a reduction on the number of fractionation steps, decreasing the need of further use of aggressive organic solvents.

Although chemically characterized by their hydrophobicity, a large spectrum of solvents' polarities has been lately applied for SL extraction. According to the literature, pure methanol, a solvent with high relative polarity (0.762, in a scale where water is assumed to have a relative polarity of 1), is the most used solvent for SL extraction and quantification (Figure 2.2 a). On the other polarity extreme, n-hexane (0.009), with low chemical affinity to SL has typically been applied on sequential extraction with increasing solvent polarities, for a first biomass defatting. In order to increase selectivity and/or decrease environmental impact, solvents' mixtures can be strategically used. Although ethanol on its pure form was not the most frequent extraction solvent applied, its aqueous solutions had become of high interest for SL extraction (Figure 2.2 b).

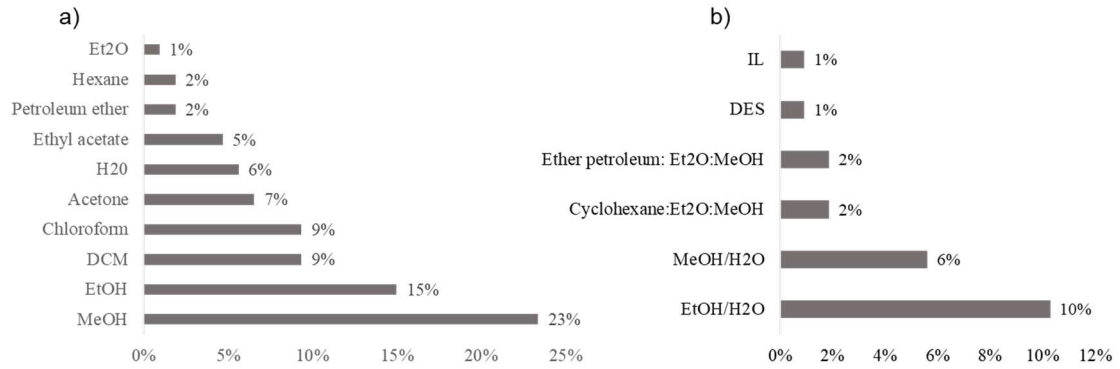


Figure 2.2. Percentage of the number of published papers from a total of 145, as a function of the solvents applied for sesquiterpene lactones extraction, using conventional extraction methodologies, during the last 10 years (2010-2019), where a) corresponds to solvents on their pure form; and b) to mixtures of solvents. H_20 - water; DCM - dichloromethane; EtOH – ethanol; MeOH - methanol; Et_2O - diethyl ether; DES - deep eutectic solvent; IL - ionic liquid aqueous solution.

During the last decade of research, as summarized in Table 2.1, the three SLs with large prevalence were artemisinin, alantolactone and cynaropicrin and were extracted by a broad range of solvents, either pure or in mixtures. Recently, new approaches using novel solvents were described for SL extraction, such as ionic liquids and deep eutectic solvents [115, 116], although extraction yields achieved with these solvents did not overcome previous results obtained with the organic solvents tested.

Table 2.1. Solvents and solvent mixtures applied for artemisin, alantolactone and cynaropicrin extraction, during the last 10 years (2009-2019). DCM – dichloromethane; EtOH – ethanol; MeOH – methanol; SC-CO_2 – supercritical carbon dioxide; H_20 - water.

Sesquiterpene lactone	Artemisin	Alantolactone	Cynaropicrin
Plant specie	*Artemisin annua* L. *Artemisia siberi*	*Inula helenium* L. *Inula oculus-christi* L. *Inula racenosa* *Inula viscosa*	*Cynara cardunculus* *Centaurea drabifolia* *Psephellus sibiricus*
Solvent	n-hexane [144] DCM [114] EtOH [144] SC-CO_2 [101] Hexane with % 1 mol ethyl acetate [144] n-hexane: ethyl acetate (95:5) [103] 96% EtOH [98]	Chloroform [127] EtOH [92, 125] H_2O [94] 95% EtOH [96] EtOH:H_2O (50:50) [92]	n-hexane [36, 115, 116] DCM [29, 37, 115, 116, 122, 145] Ethyl acetate [118, 122] Acetone [115, 116] EtOH [41, 118, 122] MeOH [36, 43, 117, 118] H_2O [116-118] Ionic Liquids [115] Deep Eutectic Solvents [119] MeOH (70%) [117] EtOH (80%) [117]

Artemisinin is one of the most well studied SL, mainly due to its great antimalarial potential, which can be found on the aerial parts of Artemisia species, for example Artemisia annua L. [146-148]. Nevertheless, other SL can be isolated/extracted from Artemisia species aerial parts, such as absinthin, artemisetin, absinthinin and hydroxypelenolide, isolated from Artemisia absinthium [99, 100, 107]. A comparison between the referred studies, clearly demonstrated that the use of different solvents allows the identification of different SL. As example, absinthin and artemisitin were extracted with ethanol 96% (v/v) [100], absinthinin by distilled water [99] and hydroxypelenolide with acetone [107].

Other three studies regarding the identification of compounds from *Hedyosmum brasiliense* leaves, applied water [149] and ethanol [150, 151] as extraction solvents, followed by extensive fractionation and purification. A comparative analysis revealed that the ethanolic extracts led to a very similar extraction yield (38 and 42 g extract/ kg fresh leaves), with podoandin as the major SL identified (1.43 and 1.58 mg/g extract) [150, 151]. On the other hand, the use of water as extraction solvent, results in an extraction yield of 15.5 g extract/ kg fresh *Hedyosmum brasiliense* leaves, with a podoandin content of 0.59 mg/g extract [149]. The analysis of these independent studies clearly underlies the importance of extraction solvent adequacy, towards an optimized extraction yield. Genta and co-authors, within a different study, used two distinct solvents to extract the SL enhydrin from *Smallanthus sonchifolius* leaves. Firstly methanol was chosen, with an extensive purification step, leading to an enhydrin content of 52 mg from a 122 mg SL reaction; in the same study, *Smallanthus sonchifolius* leaves were also extracted with chloroform achieving a 291 mg of pure enhydrin [152]. Another study using dichloromethane (DCM) as extraction solvent for enhydrin from *Smallanthus sonchifolius* leaves, led to a final SL fraction of 20 mg, with an enhydrin concentration of 7.15 ppm [153]. It is clear that solvent selection can allow not only an increase of SL extraction yield, but also a correct identification of SL present in the plant, offering the possibility to a similar tuning of solvents and mixtures of solvents.

Based on this, a new approach for selection of SL extraction solvent was recently studied by Bras et al. considering the Hansen solubility parameters (HSP) [122]. The HSP analysis was based on the concept that total cohesive energy density is approximated to the sum of the energy densities required to overcome atomic dispersion forces (δ_d^2), molecular polar forces arising from dipole moments (δ_p^2) and hydrogen-bonds between molecules (δ_h^2). In this approach, SL solubility was compared using conventional BE with ethanol, ethyl acetate, dichloromethane, ethanol/water (40/60) and water as extraction solvents. The application of Hansen solubility parameters to quantify solute/solvent interactions was established, with HSP demonstrating its potential as a tool for solute solubility determination in SL extraction from *Cynara cardunculus* leaves [122].

Besides an increase on extraction yield and/or extract concentration, extraction solvent selection could also increase the extract biological potential, as shown for extracts from the aerial parts and roots from *Tanacetum chiliophyllum* var. monocephalum grierson using hexane, ethyl acetate and methanol. The SL 1-epi-chiliophyllin was firstly identified when using ethyl acetate, obtaining a

higher DPPH scavenging activity in root extracts at 10 mg/mL concentration (87.8 ± 0.84%), comparatively to the methanolic one (83.0 ± 0.84%,) what, according to the authors, is related to the SL content [93].

2.4. Extraction methodology

Several disadvantages can be identified as major potential setbacks on solvent extraction, such as solvent toxicity, degradation of target compounds, high time consumption, low yield and low process selectivity which, besides from being directly linked to the quality and purity of the final product, are also related with environmental pollution and process costs. The appropriate extraction method selection should be established by the polarity of the constituents of interest, as well as by the partitioning equilibrium between biomass and solvent [144].

2.4.1. Conventional extraction methodologies

With a main focus on identification, rather than quantification, during the last 10 years conventional extraction methodologies have been mainly applied for SL extraction, as it is presented on Figure 2.3 and Table 2.2. Conventional extraction methodologies applied to SL extraction, such as maceration, Soxhlet (or reflux) or percolation require the use of toxic organic solvents, are time consuming (for example 528 h as referred by Shoaid et al. [112]), involve high amounts of solvents per mass of original natural matrix and compound degradation due to high temperatures are prone to occur [154].

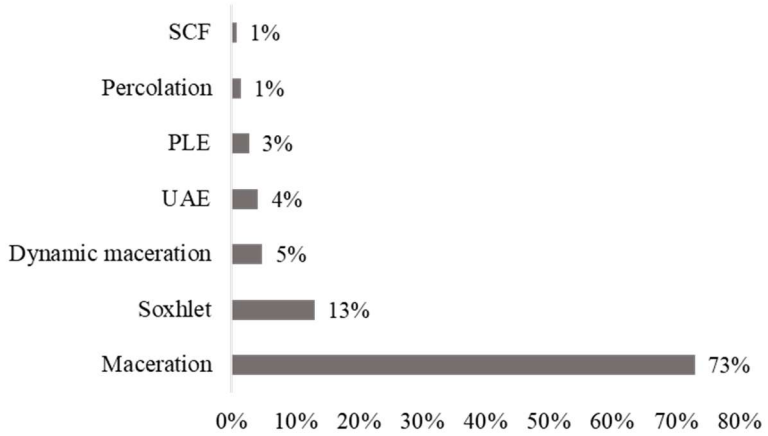


Figure 2.3. Percentage of different extraction methodologies applied for SL extraction, from a total of 145 published papers during the last 10 years (2010-2019) SCF - Supercritical fluids; UAE – Ultrasound assisted extraction; PLE – Pressurized liquid extraction.

According to the literature, there is still a lack of knowledge regarding standardization of several SL extraction parameters, such as temperature, extraction time and/or solvent/biomass ratio.

Table 2.2. Sesquiterpene lactones extracted by conventional extraction methodologies, organized by plant species during the last 10 years (2009-2019) and corresponding extraction solvent and conditions, when available. DCM – dichloromethane; EtOH – ethanol; MeOH - methanol; SC-CO_2 – supercritical carbon dioxide; Et_2O - diethyl ether; N.i. - not identified; RT - room temperature.

Plant	Part of plant	Extracted SL	Solvent	Extraction methodology	Ref.
Adansonia digitata (Mbamburi) Canthium glaucum (Mhonga) Launaea cornuta (Mtsungawautsunga) Zanthoxylum chalybeum (Mjafari/mporojo)	Stem bark roots leaves	N.i.	MeOH: Chloroform (1:1) H_2O	Immersion at 60 ºC for 1 h Maceration for 24 h (4 times)	[155]
Ainsliaea acerifólia	aerial parts	Ainsliaside C	EtOH 70%	Maceration at RT (3 times)	[156]
Ajania przewalskii	whole plant	Ajaniaolide A 1b-hydroxyeudesm-4(15)-en-5a,6b,7a, 11bH-12,6-olide 1b-hydroxyeudesm-3-oxo-4b,5a,6b,7a,11aH-12,6-olide 8a-angeloyloxyl-3a,4a-dihydroxyguaia-1,9,11(13)-trien-6,12-olide	Petroleum Eter:MeOH (1:1:1)	Maceration at RT for 96 h (3 times)	[157]
Amberboa ramosa	whole plant	Amberbin C Amberin Amberbin A Amberbin B	MeOH	Maceration at RT (3 times)	[158]

Ambrosia cumanensis Kunth	aerial parts	4α,10β-dihydroxypseudoguaian-12,6-olide 4α-hydroxy-11(13)-pseudoguaien-12,6-olide 2,3-dehydropsilostachyn C	MeOH	Maceration at RT for 48 h (3 times)	[159]
Anthemis melanolepis	aerial parts	Anthemin A 1R-hydroxydeacetylirinol-4R,5-epoxide Anthemin C Tatridin A 1-epi-tatridin B Anthemin B 6-deacetyl--cyclopyrethrosin Elegalactone A 1,4R,6Rtrihydroxyeudesm-11-en-8R-12-olide	cyclohexane-Et_2O-MeOH (1:1:1; extract A) MeOH-H_2O (1:1; extract B), successively	Maceration at RT	[160]
Anthemis rumelica (Velen.) Stoj. & Acht	flower heads	4a,9a-Diacetoxyguaia-2,1(10), 11(13)-trien-12,6a-olide 1a,4b-Dihydroxy-11aH-guaia-2,10(14)-dien-12,6a-olide Tanacin Hanphyllin	Chloroform	Maceration	[161]
Anthemis wiedemanniana Fisch. & Mey	flowers	Tatridin A Tanachin (1-epi-tatridin B)	Hexane Ethyl acetate MeOH	Sequential maceration sequential at RT	[162]
Anvillea radiata Coss. & Dur.	aerial parts	9α-hydroxyparthenolide, 9β-hydroxyparthenolide	MeOH/H_2O (7:3)	Maceration at RT	[163]
Artemisia absinthium	aerial parts	Absinthin Artemisetin Dihydro-epi-deoxyarteannuin B	EtOH 96%	Maceration for 24 h	[100]

Artemisia absinthium	whole plant	Hydroxypelenolide	Acetone	Maceration at RT for 48-72 h	[107]
Artemisia absinthium L.	aerial parts	Absinthin Anabsinthin	H_2O	Maceration for 12 h followed by boiling for 1h	[99]
Artemisia annua L.	leaves	Artemisin	DCM	Maceration with stirring for 24 h at RT	[114]
Artemisia annua L.	leaves	Artemisin	EtOH; Hexane; Hexane with 5% mol of ethyl acetate		[144]
Artemisia annua L.	leaves	Artemisin	n-hexane:ethyl acetate (95:5)	Maceration with continuous stirring for 90 min; Ultrasound assisted extraction	[103]
Artemisia anómala	whole plant	8α-acetoxy-1,10α-epoxy-2-oxo-guaia-3,11(13)-dien-12,6α-olide 13-acetoxy-1-oxo-4α-hydroxy-eudesman-2(11)-dien-12,6α-olide	95% EtOH	Maceration for 72 h (3 times)	[109]
Artemisia douglasiana Besser	aerial parts	Dehydroleucodine	Chloroform	Boiling	[164]
Artemisia korshinskyi	aerial parts	Herbolides A and B	Chloroform	Maceration	[111]
Artemisia macrocephala	aerial parts	N.i.	MeOH	Immersion with occasional stirring for 528 h (3 times)	[112]
Artemisia roxbughiana	leaves	roxbughianins A and B	MeOH	Maceration at RT, for 72 h (3 times)	[110]

Artemisia scoparia Waldst. & Kit.	aerial parts	Scoparanolide	H_2O	Autoclave for 30 min	[105]
Artemisia sieberi	aerial parts	Artemisin	96% EtOH	Maceration at RT for 72 h (3 times)	[98]
Asparagus falcatus (Linn.)	leaves	Aspfalcolide	Chloroform	Shaking in vortex	[165]
Aucklandialappa Decne	roots	Costunolide Dehydrocostuslactone	MeOH	Reflux for 2 h	[166]
Calea pinnatifida	leaves	Calein C Calealactone C	MeOH	Maceration at RT	[24]
Calea urticifolia DC.	leaves	2,3-epoxyjuanislamin, Calealactone B, Calein C	Chloroform	Maceration for 72 h	[167]
Carpesium abrotanoides	fruits	9-hydroxy-1H, 11H-guaia-4,10(14)-dien-12,8-olide 9-hydroxy-1H, 11H-guaia-4,10(14)-dien-12,8-olide	MeOH	Maceration at RT for 168 h	[168]
Carpesium abrotanoides	whole plant	5α-hydroxy-4α,15-epoxy-11αH-eudesman-12,8β-olidecarabrol-4-O-palmitatecarabrol-4-O-linoleate	95% EtOH	Maceration at RT for 168 h	[169]
Carpesium humile	aerial parts	Carpelipine Divaricin B	Ether petroleum/Et_2O/MeOH (1:1:1) 75% EtOH	Maceration followed by reflux at RT	[170]
Carpesium macrocephalum	whole plant	Carpedilactones E–G	Acetone	Maceration at RT (5 times)	[171]

Centaurea drabifolia subsp. Detonsa	aerial parts	Aguerin B Cynaropicrin 8α-isovaleryloxyzaluzanin C 8α-acetoxyzaluzanin C 4β,15-dihydro-3-dehydrosolstitialin A monoacetate	n-hexane MeOH	Sequential maceration (40 h)	[36]
Centaurea pannonica	aerial parts	2a-hydroxy, 8-dehydroxy 15-O-methacrylate salonitenolide 2a,8a-dihydroxy-dehydrocostus lactone Pannonin	cyclohexane–Et_2O–MeOH (1:1:1; extract A) MeOH–H_2O (1:1; extract B)	Maceration at RT	[172]
Chandonanthus hirtellus	whole plant	Chandolide	Et2O	Maceration at RT for 168 h (3 times)	[173]
Chartolepsis Intermedia Boiss.	aerial parts	Grosheimin Cynaropicrin	EtOH	Dynamics maceration for 2 h	[41]
Chloranthus elatior	whole plant	Chlorantholides A–F	EtOH:H_2O (95:5)	Maceration at RT 240 h (3 times)	[174]
Chloranthus multistachys	aerial parts	Eremophilanolide Intanbulin A	EtOH	Reflux for 2 h (2 times)	[175]
Chlorantus japonicus	whole plant	Chlojaponilactones F-I	EtOH 95%	Reflux 3 times	[176]
Chlorantus serratus	roots	Chloranthalactone Eserralactone A 8β, 9α-dihydroxylindan-4(5), 7(11)-dien-8α, 12-olide	EtOH	Reflux for 2 h (3 times)	[86]
Chrysophthalmum montanum (DC.)	aerial parts	6α-acetoxy-4α- hydroxy-1βH-guaia-9.11(13)-dien-12.8α-olide 6α-acetoxy-4α-hydroxy-9β.10β-epoxy-1βH-guaia-11(13)-en-12.8α-olide 4α,6α-dihydroxy-1β,5α,7αH-guaia-9(10),11(13)-dien-12,8α-olide	MeOH 80%	Maceration with stirring at RT for 48 h (4 times)	[177]

		(4α,5α,8β,10β)-4,10- dihydroxy-1,11(13)-guaidien-12,8-olide			
Cichorium endivia L. var. crispum Cichorium endivia L. var. latifolium	aerial parts	11(S),13-Dihydrolactucin Lactucin 8-Deoxylactucin 11(S),13-Dihydro-8-deoxylactucin 11(S),13-Dihydrolactucopicrin Lactucopicrin	2% formic acid in $MeOH/H_2O$ (4/1)	Maceration	[178]
Cichorium intybus L.	whole plant	Lactucopicrin-15-oxalate Chicoralexin	Petroleum ether, chloroform, ethyl acetate and MeOH	Soxhlet for 8 h	[179]
Crepis incana Sm	aerial parts	Grosheimin Crepiside E, Crepiside D nor-isoprenoid (3S,5R)-loliolide	cyclohexane:Et_2O:H_2O (1:1:1) $MeOH:H_2O$ (5:1)	Sequential maceration	[180]
Cyathocline purpúrea	aerial parts	6α-hydroxy-4 [14], 10 [15]-guainadien-8α, 12-olide (HGN)	MeOH	Percolation at RT for 12 h (3 times)	[181]
Cynara cardunculus L.	leaves	Cynaropicrin	Ionic liquids DCM n-hexane	Maceration 25 ºC for 2 h	[115]
Cynara cardunculus L.	leaves	Cynaropicrin	DCM	Soxhlet for 7 h	[37]
Cynara cardunculus L.	Leaves	Cynaropicrin	Deep Eutectic solvents DCM n-hexane Acetone H_2O	Maceration 25 ºC for 2 h Soxhlet for 7 h	[116]

Cynara cardunculus L. var. altilis DC	whole plant	Cynaropicrin Grosheimin Deacylcynaropicrin	DCM	Soxhlet for 7 h	[29]
Cynara cardunculus L. var. altilis DC.	leaves	Cynaropicrin	H_2O EtOH (80%) MeOH (70%)	Maceration at RT for 72 h	[117]
Cynara cardunculus var. altilis	leaves	Cynaropicrin; Grosheimin; Aguerin B	MeOH EtOH Ethyl acetate H_2O	Immersion	[118]
Cynara cardunculus var. scolymus	leaves	Cynarinin A Cynarascoloside C Aguerin B	MeOH	Maceration at RT	[182]
Cynara cardunculus	leaves	Cynaropicrin	Ethanol Ethyl acetate H_2O Ethanol/ H_2O /(40/60)	Stirring at 30 ºC, 900 rpm for 1 h	[122]
Drimys winteri	bark	Drimenin	Ethyl acetate	Maceration for 168 h	[183]
Elephantopus mollis	herbs	8-O-methacryloylelephanpane 2,4-bis-O-methyl-8-O-methacryloylelephanpane 4-O-ethyl-8-O-methacryloylelephanpane 8-O-methacryloylisoelephanpane 2-Odemethyltomenphantopin C Molephantin A Molephantin B	EtOH:H_2O (95:5)	Maceration at 70 ºC (3 times)	[184]
Eupatorium perfoliatum	aerial parts	N.i.	DCM	Soxhlet extraction	[185]

Eupatorium perfoliatum L	whole plant	2-oxo-8-tigloyloxyguaia-1(10),3-diene-6,12-olide-14-carboxylic acid	MeOH/ H_2O (70/30)	Rotor-stator extraction (3 min) followed by Ultra-Turrax	[186]
Ferula varia	roots	N.i.	MeOH	Maceration at 60 ºC for 4 h (3 times=	[89]
Gynoxys verrucosa	aerial parts	Leucodine Dehydroleucodine	Ethyl acetate	Dynamic maceration at RT for 5 h	[187]
Gynoxys verrucosa	aerial parts	Dehydroleucodine Leucodine	Ethyl acetate	Maceration at RT	[188]
Hedyosmum brasiliense	leaves	1- a-acetoxyeudesma-3,7(11)–dien–8,12–olide	H_2O	Infusion at 100 ºC for 30 min	[149]
Hedyosmum brasiliense	leaves	13-hydroxy-8,9-dehydroshizukanolide Podoandin Elemanolide 15-acetoxy- Isogermafurenolide	EtOH	Bi-distillation for 360 h (3 times)	[151]
Hedyosmum brasiliense	leaves	1,2-epoxy-10a-hydroxy-podoandin 1-hydroxy-10,15-methylenepodoandin 15-acetoxy-isogermafurenolide 8a/b,9ahydroxy-onoseriolide Podoandin Onoseriolide 15-hydroxy-isogermafurenolide	EtOH:H_2O (95:5)	Maceration at RT for 360 h	[150]
Helianthus annus	florets	Argophyllone B, Niveusin B, 15hydroxy-3-dehydrodesoxyfruticin	DCM	Vortex	[189]

Helianthus tuberosus L.	leaves	3-Hydroxy-8b-tigloyloxy-1,10-dehydroariglovin	Ethyl acetate	Maceration overnight (3 times)	[190]
Hieracium species	flowering heads	Calophyllamine B Calophyllamine A 8-Epiixerisamine A Crepiside E Desacylcynaropicrin	DCM MeOH	Maceration at RT for 48 h Bimaceration at RT for 48 h (2 times)	[191]
Hosta ensata	whole plant	Hostasolide A	MeOH	Maceration (3 times)	[192]
Hymenoxys robusta	aerial parts	Vermeerin	Chloroform	Maceration at RT for 60 h days	[193]
Illicium burmanicum	stem bark	Burmanicumolide A Burmanicumolide B Burmanicumolide C Burmanicumolide D	80% MeOH	Reflux for 2 h (3 times)	[194]
Illicium henryi	stems	Illihenlactone A	EtOH/H2O (95/5)	Reflux at 90-95 ºC 2 h (3 times)	[195]
Illicium difengpi	stem bark	Difengpilactone	DCM and MeOH	Sequental maceration	[196]
Innula cappa	aerial parts	Ineupatolides A–C	Acetone	Maceration at RT	[128]
Inula britannica	flowers	14-(3-Methylpentanoyl)-6-deoxybritannilactone 14-(3-Methylbutanoyl)-6-deoxybritannilactone 1,3-Epi-granilin 11,13-Dihydro-inuchinenolide B	EtOH	Maceration for 24 h	[123]
Inula cappa	whole plant	Ineupatolides D and E	95% EtOH	Maceration RT for 7 h (3 times)	[130]

Inula helenium L.	roots	Alantolactone Isolantolactone Diplophyllin	H_2O	Steam distillation	[94]
Inula helianthus-aquatica	leaves and flowers	Bigelovin	Petroleum ether	Successively extracted	[131]
Inula hupehensis	aerial parts	Rufesolides A Rufesolides B Rufesolide C Rufesolide D Rufescenolides A Rufescenolides B	95% EtOH	Maceration at RT for 24 h (3 times)	[197]
Inula japonica Thunb	flowers	JEUD-38	75% EtOH	Reflux for 2 h (3 times)	[129]
Inula montana L.	leaves and flowers	Artemorin	95% EtOH	Percolation at RT for 18 h	[124]
Inula oculus-christi L.	aerial parts	4H-tomentosine Alantolactone Hydroxyalantolactone Epi-granilin	Chloroform	Maceration at RT	[127]
Inula racemosa	roots	1-one-4-epi-alantolactone 4a,13-dihydroxy-5,7(11)-eudesmadien-12,8-olide	95% EtOH	Reflux for 3 h (3 times)	[96]
Inula racenosa	roots	Alantolactone Isolantolactone	EtOH; H_2O and EtOH/H_2O 50%	Sequential Maceration for 10 h by maceration at 40 ºC for 40 h and then continuous stirring mixture at 25 ºC for 24 h	[92]

Inula viscosa	leaves	Inuviscolide 4H-tomentosine Alantolactone Hydroxyalantolactone epi-granilin	EtOH	Continuous stirring at RT for 24 h	[125]
Ixeris sonchifolia	whole plant	Sonchifoliactone A1	H_2O	Maceration for1 h (3 times)	[198]
Lactuca aculeata	aerial parts	Crepidiaside B 8-deoxylactucin Jacquinelin 9a-hydroxy-zaluzanin C and its 11b, 13-dihydroderivative macrocliniside A ixerin F glucozaluzanin C and its 11b, 13-dihydroderivative Lactuside A	EtOH	Continuous stirring at RT	[199]
Lactuca canadensis	roots	11,13-dehydrolactuside C 3-epizaluzanin C-3-O-b-glucopyranoside zaluzanin C and its 11b,13-dihydroderivative 3-epizaluzanin C and its 11b,13-dihydroderivative vernoflexuoside (glucozaluzanin C) and its 11b,13-dihydroderivative Macrocliniside A Ixerin F Picriside B santamarin and its 11b,13-dihydroderivative Armexifolin 1-epidehydroisoerivanin Armefolin	EtOH	Continuous stirring at RT	[91]

		1-epiisoerivanin 3a-hydroxyreynosin 1-epierivanin			
Lactuca geórgica	leaves and roots	Cichorioside B Lactucin 11b,13-dihydrolactucin 11b,13dihydrolactucin-8-O-acetate 11b,13-dihydrolactucopicrin 11b,13-dihydrolactucin-8-O-methacrylate Lactuside A	MeOH	Maceration	[85]
Lactuca sativa L var. anagustata	stalks	1b-O-b-D-glucopyranosyl-4ahydroxyl-5a, 6b, 11bH-eudesma-12, 6a-olide 1b-hydroxyl-15-O-(p-methoxyphenylacetyl)-5a, 6b, 11bH-eudesma-3-en-12, 6a-olide 4a-O-b-D-glucopyranosyl-15-hydroxyl-5a, 6bH-guaiane-10(14), 11(13)-dien-12, 6a-olide 9b-hydroxyl-4b, 11b, 13, 15-tetrahydrozaluzanin C 10b, 14-dihydroxyl-11bH-guaiane-4(15)-ene-12, 6a-olide 1b-hydroxyl-5a, 6bH-eudesman-3-ene-12, 6a-olide macroclinisides A 11b, 13-dihydrolactucin Cichorioside B 11b, 13-dihydrolactucopicrin 10b, 14-dihydroxy-10(14), 11b(13)-tetrahydro-8, 9-didehydro-3-	MeOH	Maceration at RT for 7 days	[200]

		deoxyzaluzaninC-10-O-b-Glucopyranoside			
Lactuca sativa var. capitate	heads	Cichorioside B 8-deacetylmatricarin-8-O-sulfate 11ß,13-dihydroxy-lactucin-8-O-sulfate	MeOH:H_2O (85:15)	Maceration at 4 ºC for 24 h	[201]
Laserpitium species	underground parts	2b,8a-diangeloyloxy-10b-hydroxy-6aH-guaian-3,(7-11)-dien-12,6-olide 8a-acetoxy-2b,10b-diangeloyloxy-6aH,7aH-guaian-3-en-12,6-olide 8a-Angeloyloxy-11a-senecioyloxy-6aH,7aH-guaian-3-en-12,6-olide 2b,8a-di-Angeloyloxy-10b-hydroxy-6aH-guaian-3,(7-11)-dien-12,6-olide 8a-acetoxy-2b,11a-diangeloyloxy-10b-hydroxy-6aH,7aH-guaian-3-en-12,6-olide 10b-acetoxy-8a-angeloyloxy-6aH,7aH-guaian-3-en-12,6-olide Isosilerolide 8a-senecioyloxy-10b-hydroxy-6aH,7aH-guaian-3-en-12,6-olide	Chloroform	Maceration for 48 h (2 times)	[202]
Launaea nudicaulis	whole plant	Nudicholoid	MeOH	Maceration (3 times)	[203]
Laurus nobilis L.	leaves	Reynosin Hydroperoxide-magnolialide 1b,2b-dihydroxy-5a,6b,7aH-eudesma-4(15),11(13)-dien-12,6-olide Santamarine Magnolialide 3a-peroxyarmefolin 13-dehydrosantonin Tubiferin	DCM and MeOH	Maceration for 48 h (2 times)	[204]

		Anhydroperoxycostun-olide Lucentolide 3aS,5aR,6S,7R,9aR,9bS)-6-hydroxy-7-acetoxy-5a-methyl-3,9-dimethylidene-3a,4,5,6,7,8,9a,9b-ctahydrobenzo[g][1]benzofuran-2-one 3aS,5aR,6R,7R,9aS,9bS)-6,7-dihydroxy-5a,9-dimethyl-3-methylidene-4,5,6,7,9a,9b-hexahydro-3aH-benzo[g][1]benzofuran-2-one 3aS,5aR,6R,7R,8R,9S,9aS,9bS)-6,7,8,9-diepoxy-5a,9-dimethyl-3-methylidene-5.3a,4,5,6,7,8,9a,9b-octahydrobenzo[g][1]benzofuran-2-one 3aS,5aR,6R,9S,9aS,9bS)-6,9-dihydroxy-5a,9-dimethyl-3-methylidene-6.3a,4,5,6,7,8,9a,9b-octahydrobenzo[g][1]benzofuran-2-one 3aS,5aR,6R,9R,9aS,9bS)-6-hydroxy-9-methoxy-5a,9-dimethyl-3-methylidene-3a,4,5,6,7,8,9a,9b-octahydrobenzo[g][1]benzofuran-2-one			
Ligularia hodgsonii Hook	roots and rhizomes	Biliguhodgsonolide	95% EtOH-H_2O	Maceration at RT	[88]
Lindera strychnifolia	roots	Linderolides A–F	MeOH	Maceration at RT for 168 h (3 times)	[95]
Lindera strychnifolia	roots	Linderolide G–M	MeOH–H_2O (4:1)	Maceration (3 times)	[90]
Lychnophora trichocarpha	whole plant	Lychnopholide Eremantholide C	EtOH	Maceration at RT	[205]

Mikania micrantha Mikania cordifolia	aerial parts	8-epi-mikanokryptin Melampolide 11Hβ-11,13-dihydromicrantholide Dihydromikanolide Deoxymikanolide Miscandenine Achalensolide Xerantholide 15-O-3′-hydroxy-isobutyryl-micrantholide 15-O-4′-hydroxy-methacryl-micrantholide 15-O-4′-hydroxymethacryl-14-acetoxy-micrantholide 15-O-3′-chloro-2′-hydroxy-isobutyryl-micrantholide 15-O-2′-hydroxyisobutyryl-micrantholide Micrantholide	n-hexane followed by DCM/MeOH (1/1)	Maceration at RT with for both solvents (3 times) solvent exchange. Second extraction for 48 h	[206]
Neolitsea kedahensis Gamble	stems	Pseudovillosine	Hexane Ethyl acetate	Sequential maceration at RT	[207]
Neurolaena lobata (L.) R. Br. ex Cass.	leaves	Neurolenins B, C + D Lobatin B 9a-hydroxy-8b-isovalerianyloxy-Calyculatolide	EtOH:H_2O (4:1)	Maceration at RT for 24 h	[208]
Onopordum acanthium L.	leaves	Onopordopicrin	Ethyl acetate	Maceration for 24 h	[209]
Parasenecio roborowskii	aerial parts	Parasenolide A-F (6S,8S,10S,14R, 15R)-8,10-dihydroxy-6-methoxyleremophilenolide	Ether petroleum/Et_2O/MeOH (1:1:1) followed by reflux with 75% EtOH	Maceration at 18 ºC for 432 h) followed by boiling with EtOH for 16 h	[210]

Parthenium hysterophorus L.	aerial parts	Parthenin	MeOH	Maceration for 24 h	[211]
Pimpinella haussknechtii	fruits	Pimpinelol	Chloroform	Maceration for 72 h	[21]
Piptocoma rufescens	leaves	Rufescenolides A-B Rufesolide D 5-epiisogoyazensolide Goyazensolide Rufesolide A-D 15-deoxygoyazensolide	MeOH	Maceration at RT	[197]
Pseudelephantopus spicatus	aerial parts	(+)-8,13-Diacetyl-piptocarphol (+)-8-Acetyl-13-O-ethyl-piptocarphol	EtOH	Maceration at RT (4 times)	[212]
Pseudelephantopus spiralis (Less.)	aerial parts	Piptocarphol Piptocarphins A	H_2O	Boiling for 10 min	[213]
Pseudelephantopus spiralis (Less.) Cronquist	aerial parts	Spiraloside 1a,10a-epoxy-8a,13-diacetylpiptocarphol 5b-(2-ethylacryloyloxy)spicatocadinanolide A	MeOH	Maceration at RT for 38 h each (3 times)	[214]
Rolandra fruticosa	leaves and twigs	isorolandrolide, 13-ethoxyisorolandrolide bourbonenolide, 2a,13-diacetoxy-4a-hydroxy-8a-isobutyroyloxybourbonen-12,6a-olide, 13-acetoxyrolandrolide, 8-desacyl-13-acetoxyrolandrolide-8-O-tiglate, 2-epi-glaucolide E, 2a,13-diacetoxy-4a-hydroxy-8a-methacryloyloxybourbonen-12,6a-olide, 2a,13-diacetoxy-4ahydroxy-8a-tigloyloxybourbonen-12,6a-olide	MeOH	Maceration at RT for 24 h (3 times)	[215]

S. indicus	flowers and frutis	7-hydroxyfrullanolide	MeOH	Stirring at 60 ºC for 3 h	[216]
Saussurea involucrata	aerial parts	Sausinlactones A-C 8α-dihydroxyl-11βH-11, 13-Dihydrodehydrocostuslactone 8α-hydroxyl-11βH-11,13-Dihydrodehydrocostuslactone 11β,13-dihydrodehydrocostuslactone-8-O-β-D-glucoside 11β,13-dihydrodehydrocostuslactone-8-O-[6′-O-acetyl-β-D-glucoside] 11α,13 -dihydroglucozaluzanin C Japonicolactone	95% EtOH	Reflux for 3 h (3 times)	[217]
Scorzonera austriaca	roots	Scorzoaustriacoside Scorzoaustriacin Scorzoaustriacin 3-O-β-d-glucoside 4-epi-dihydroestafiatol 14-isovaleroxyscorzoaustricin 14-isovaleroxyscorzoaustricin sulfate	Acetone followed by 95% EtOH	Sequential maceration (acetone) (4 times) and (ethanol) (2 times)	[157]
Senecio kingii Hook	aerial parts	1a-angeloyloxy-8b-methoxy-10bH-eremophil-7(11)-en-8,12-olide) 1a-angeloyloxy-8bH,10bH-eremophil-7(11)-en-8,12-olide 1a-angeloyloxy-6b-hydroxy-8bmethoxy-10bH-eremophil-7(11)-en-8,12-olide 1a-tigloyloxy-8bH,10bH-eremophil-7(11)-en-8,12-olide 1a-angeloyloxy-6b,8b-dihydroxy-10bHeremophil-7(11)-en-8a,12-olide	MeOH	Maceration at RT for 168 h	[218]

		1a-angeloyloxy-6b,8b-dimethoxy-10bH-eremophil-7(11)-en-8a,12-olide 1a-angeloyloxy-6b-hydroxy-8b,10bH-eremophil-7(11)-en-8a,12-olide 1a-tigloyloxy-8b-methoxy-10bH-eremophil-7(11)-en-8a,12-olide 1a-tigloyloxy-6b,8b-dihydroxy-10bH-eremophil-7(11)-en-8a,12-olide 1a-tigloyloxy-6b-hydroxy-8bH,10bH-eremophil-7(11)-en-8a,12-olide 1a-tigloyloxy-6b-hydroxy-8b-methoxy-10bH-eremophil-7(11)-en-8a,12-olide			
Smallanthus sonchifolius	leaves	Enhydrin	MeOH	Maceration 120 h (3 times)	[152]
Smallanthus sonchifolius	leaves	Uvedalin Enhydrin Polymatin B Sonchifolin Fluctuanin	DCM	Maceration at RT for 30 min (2 cycles)	[219]
Smallanthus sonchifolius	leaves	Enhydrin Uvedalin	DCM	Maceration for 15 min	[153]
Sonchus arvensis	whole plant	1β,15-diacetoxy-5,7α,6,11β(H)-eudesm-3,4-en-6,12-olide 1β-hydroxy-3,4-en-15-O-β-glucopyranosyl-5,7α,6,11β(H)-eudesman-6,12-olide	85% EtOH	Reflux for 3 h (3 times)	[220]
Sonchus arvensis L. (Asteraceae)	whole plant	1β-sulfate-5α, 6βH-eudesma-3-en-12, 6α-olide 1β-(p-hydroxyphenyl acetyl)-15-O-β-D-	85% EtOH	Reflux for 3 h (3 times)	[221]

		glucopyranosyl-5α, 6βH-eudesma-3-en-12, 6α-olide			
Sphaeranthus indicus	aerial parts	Indicusalactone (-)-oxyfrullanolide	n-hexane:ethyl acetate: MeOH	Maceration at RT (3 times)	[222]
Stizolophus balsamita ***Psephellus sibiricus***	leaves	cynaropicrin desalcynaropicrin (*P. sibiricus*) Balsamin, Izospiciformin, Stizolin, 9α-hydroxyparthenolide, 8α-E-(4′-hydroxy)-senecioyloxy-9α-hydroxyparthenolide, stizolicin, 11βH,13-dihydrostizolicin (*S. balsamita*)	MeOH	Immersion	[43]
Tanacetum chiliophyllum (Fisch. & Mey.) var. monocephalum grierson	aerial parts and roots	1-epi-chiliophyllin	hexane ethyl acetate MeOH	n.i.	[93]
Tanacetum oshanahanii	aerial parts	Tatridin A Tatridin B 6-angeloyloxy tatridin A 6a-angeloyloxydesacetyl-b-cyclopyrethrosin 6a-angeloyloxy tatridin B Tamirin Desacetyl-b-cyclopyrethrosin Tanapsin 1b,10a-epoxydesacetyllaurenobiolide 6-O-angelate	95% EtOH	Soxhlet extraction for 72 h	[134]

Tanacetum oshanahanii	aerial parts	Tanapsin	95% EtOH	Soxhlet extraction for 72 h	[132]
Tanacetum parthenium (L.)	aerial parts	N.i.	EthOH/H2O (9/1)	Dynamic stirring at RT	[133]
Telekia speciosa	whole plant	Isotelekin Inuviscolide Asperilin 2,3-Dihydroaromaticin 8-Epitomentosin Telekin Isoalantolactone	Acetone	Continuous stirring at RT for 3 h	[126]
Tithonia diversifolia	leaves	Tagitinins	Acetone	Rinsed for 20s	[19]
Vernonia amygdalina	leaves	Vernodalinol Vernodalol	MeOH	Maceration	[138]
Vernonia blumeoides	aerial parts	Blumeoidolide-A-D	n-hexane; DCM; ethyl acetate; MeOH	Sequential maceration	[136]
Vernonia cinérea	aerial parts	8a-(2-methylacryloyloxy)-hirsutinolide-13-O-acetate 8a-(4-hydroxymethacryloyloxy)-hirsutinolide-13-O-acetate, 8a-tigloyloxyhirsutinolide-13-O-acetate, 8a-(4-hydroxytigloyloxy)-hirsutinolide-13-O-acetate)	95% EtOH	Maceration at 37 ºC (3 times)	[140]
Vernonia cinérea	aerial parts	8α-(2′Z-tigloyloxy)-hirsutinolide, 8α-(2′Z-tigloyloxy)-hirsutinolide-13-O-acetate, 8α-(4-hydroxytigloyloxy)-hirsutinolide, 8α-hydroxy-13-O-tigloyl-hirsutinolide,	Chloroform	Maceration at RT (3 times)	[142]

Vernonia guineensis Benth.	leaves	Vernopicrin Vernomelitensin	Acetone	Maceration for 24 h (2 times)	[141]
Vernonia scorpioides	flowers and leaves	hirsutinolide glaucolide	EtOH	Maceration at RT for 168 h	[137]
Xanthium chinense	fruits	pungiolides A-N	EtOH (95:5)	Reflux	[223]
Xanthium sibiricum	aerial parts	11R-eremophil-1(10)-en-12,8b-olide eremophil-1(10),11(13)-dien-12,8b-olide 8-epi-xanthatin-1b,5bepoxide Tomentosin Sibiriolide C-D	95% EtOH	Maceration at RT (4 times)	[23]
Xanthium sibir-icum	aerial parts	Xanthatin	95% EtOH	Soaked at RT for 48 h	[224]

2.4.2. Non conventional extraction methodologies

Classical techniques for plant-derived biomolecules extraction are based upon correct choice of solvent coupled with the use of heat and/or agitation, which is typically translated in high energy consumptions, associated with high extraction time. The increase interest of food, cosmetics, nutraceuticals and pharmaceuticals industries for solvents residue free products (green compounds), demands for efficient, green and alternative (non-conventional) extraction methodologies, requiring short extraction time and low energy expenditure and solvents' consumption [143, 225-228].

Although research on SL extraction by non-conventional methodologies is still scarce, in the last years it has been observed an increase on the use of supercritical fluids, ultrasound assisted extraction and pressurized liquid extraction applied to SL (Table 2.3).

Supercritical fluids

Supercritical fluids (SCF) have been used in different fields such as food, pharmaceutical, chemical and oil&gas industries. This technique has the distinction of be highly selective for non-polar compounds, use a non-toxic solvent (usually CO_2) and leaving no solvent residue in the final extracted product. SCF is typically associated with high investments when compared with conventional apparatus. Nonetheless, the high cost of manufacturing is mainly attributed to limited technical/scientific knowledge, although some studies underlie that SCF can effectively be competitive for the production of bioactive extracts [229].

Although several solvents can be applied, carbon dioxide (CO_2) is the most studied, presenting as main advantages to be considered non-explosive, nontoxic, low cost and leaving no residual presence in the final product [101]. From that, and due to the ability to solubilize lipophilic substances, it turned attractive to SL extraction as an alternative to organic solvents, as shown by Baldino et al, for artemisin extraction from *Artemisia annua* L. leaves [101]. Besides, CO_2 is a gas at room temperature and atmospheric pressure, which makes the recovery of the target compounds very simple, resulting in a final solvent-free extract. In order to increase selectivity, co-solvents or modifiers are usually added to CO_2, with ethanol being the most common co-solvent used in SL extraction [135].

During the extraction of hydroxypelenolide from *Artemesia abinthium* L. leaves and roots, Martin et al [230] evidenced that SFC obtained extracts exhibited stronger antifeedant effects, up to 8 times higher than the conventional extraction methodologies.

Ultrasound assisted extraction

Ultrasound assisted extraction (UAE) is characterized by the application of sound waves between 20 kHz and 100 MHz in a liquid medium, containing the solid matrix to be extracted. The passage of waves through the liquid, promotes cavitation, derived by compression and expansion of the liquid. During cavitation, the resulting shear force mechanically breaks the sample cell wall,

improving mass transfer and accelerating solvent access to the plant cell materials [226, 228]. UAE has the advantage of extracting natural products at low temperatures, with high mass transport rates (up to 7 times higher), enhancing both extraction yield and compounds' purity [226].

UAE applied to SL extraction was firstly applied by Ferioli et al. [231], where hydrophilic mixtures of methanol/water acidified with small percentages of formic acid led to consistently higher SL recoveries in comparison to organic solvents, and enabled the simultaneous extraction of phenolic compounds. UAE upon artemisinin recovery from *Artemisia annua* L. allows for an increase of mass transport rates [113] and lower temperature and extraction time for the same extraction yield achieved with conventional methodologies. An improvement of the extraction yield (58% higher) with UAE was obtained at lower temperatures (25 ºC) promoting additionally an energy cost reduction [102].

Pressurized liquid extraction

Recently, pressurized liquid extraction (PLE), also described as accelerated solvent extraction (ASE), is becoming more popular for the extraction of natural compounds, offering advantages with respect to solvent consumption, extraction yields, extraction time and reproducibility [228, 232]. The concept of PLE is the application of high pressure to maintain the solvent in a liquid form beyond its normal boiling point. High pressure facilitates the extraction process, promoting higher analyte solubility by increasing both solubility and mass transfer. It also decreases viscosity and surface tension of solvents, thus improving extraction rates [226, 233]. Moreover, pressure may cause cell wall disruption and therefore increasing mass transfer [232].

The influence of PLE in SL extraction became a subject of interest in the last years. Ferreira et al. [106] studied artemisinin recovery from *Artemisia annua*, using different extraction methodologies, as distillation, Soxhlet and PLE. Artemisinin extraction by the combination of petroleum ether with PLE resulted in the best, and significantly highest extraction yield efficiency, while water, regardless the method used, resulted in the significantly lowest extraction efficiency. Petroleum ether combined with refluxing of ground leaves for 60 min extracted approximately 70% of the artemisinin from leaves, compared to PLE with petroleum ether. The lower efficiency of extraction with water was not improved by combining this solvent with PLE. A similar behavior was observed during Thapsigargin extraction from *Thapsia garganica* L. using acetone as extraction solvent and comparing PLE with maceration at room temperature for 12 h, with a decrease on solvent consumption and a reduced labor time with increase extraction yields [234]. PLE compared with other extraction methodologies also allows a decrease on the number of extraction steps, due to the no need of centrifugation or filtration [235], but with the disadvantage of high energy costs [122].

Table 2.3. Sesquiterpene lactones extracted from different plant species by non-conventional extraction methodologies, during the last 10 years (2009-2019. Identification of the SL extracted, the solvent used as well the extraction methodology. EtOH – ethanol; MeOH – methanol; H_20 – water; RT- room temperature; SC – Supercritical; US – Ultrasound; PLE - pressurized liquid extraction.

Plant	**Part of plant**	**Extracted SL**	**Solvent**	**Extraction methodology**	**Ref.**
Artemisia annua L.	aerial parts	Artemisin	CO_2	SC-CO_2	[101]
Cichorium inthybus L.	leaves	Lactucin, 8-deoxylactucinlactucopicrin11(S), 13-dihydrolactucin11(S), 13-dihydro-8-deoxylactucin11(S), 13-dihydrolactucopicrin	Acetone Ethyl acetate EtOH MeOH MeOH/acetone1/1 (v/v) MeOH/ethyl acetate 1/1 (v/v) 0.1%, 0.5% and 2% (v/v) formic acid in MeOH/water 4/1 (v/v)	Shaken in vortex (1 min) – sonication (10 min; RT)	[231]
Artemisia annua	leaves	Artemisin	Hexane	US bath (25 ºC,35 ºC, 45 ºC) during 15, 60 and 120 min	[102]
Cynara cardunculus	leaves	Cynaropicrin	EtOH Ethyl acetate H_2O EtOH/ H_2O (40/60)	US probe – 15% amplitude, 5 min, 40 ºC	[122]
C. intybus L. var dentatum) *radicchio "Rosso di Chioggia"* *radicchio "Rosso di Treviso Precoce"* *C. intybus L. Rubifolium Group*	leaves	11β,13-dihydrolactucin, Lactucin, 8-deoxy-lactucin, Dihydro-8-deoxylactucin, Dihydrolactucopicrin, Lactucopicrin Lactuside C (jaquinellin glucoside), Dihydro-lactucopicrin oxalate	2% formic acid in MeOH/ H_2O (4/1)	Shaken in vortex (1 min) – sonication (10 min; RT)	[236]
Xanthium spinosum L.	aerial parts	Xanthatin	H_2O	Infusion in US bath (30 min, 100 ºC)	[237]
I. helenium	roots	Alantolactone	MeOH	Sonication (120 min)	[238]

Artemisa annua	leaves	Artemisin	H_2O; Petroleum Ether	PLE - static: 5 min; flush: 100%; purge: 60 s; cycle: 3; temperature/pressure: 70 ºC/ 1500 psi	[106]
Cynara cardunculus	leaves	Cynaropicrin	EtOH Ethyl acetate H_2O EtOH/ H_2O (40/60)	PLE – static: 5 min; temperature/pressure: 40 ºC/ 10.77 kPa	[122]
Thapsia garganica L.	roots	Thapsigargin	Acetone	PLE – static: 5 min; flush: 100%; purge: 120 s; cycle: 2; temperature/pressure: 30 ºC / 100 bar	[234]
Anvillea radiata	aerial parts	9α-hydroxyparthenolide 9β-hydroxyparthenolide	Chloroform	PLE – static: 5 min; flush: 65%; purge: 120 s; cycle: 2; temperature/pressure: 40 ºC / 100 bar	[235]

2.5. Cynaropicrin as case study

Although not restricted to the *Cynara* genus, it is in *Cynara cardunculus* leaves that cynaropicrin (Figure 2.1C) is largely available as shown by the lipophilic fraction chemical characterization of several parts of *Cynara cardunculus*, described by Ramos et al. [29]. According to the same author, almost 93% of SL content on leaves (94.5 g/kg DW) is represented by cynaropicrin (87.5 g/ kg DW).

In a recent study, cynaropicrin biosynthetic pathways were studied, with *germacrene A synthase* (*GAS)*, *germacrene A oxidase* (GAO) and *costunolide synthase* (COS) genes reported as likely involved in cynaropicrin biosynthesis, with leaves of *Cynara cardunculus* exhibiting the highest expression levels of these genes [37], in agreement with the cynaropicrin content.

In recent years, cynaropicrin biological potential has raised interest due to its biological activities such as anti-inflammatory [35], cytotoxic and pro-apoptotic action against leukocyte cancer cells such as lymphoma or leukemia [26], antiopisthorchiasis [20], anti-proliferative against Triple negative breast cancer cell line (MDA-MB-231) [121], inhibition of transcription activity of nuclear factor-kappa B (NF-κB) [239], or functional downregulation of CD29- and CD98-mediated diseases such as virus-induced chronic inflammation, and invasion, migration, and metastasis of leukocyte cancer cells [25].

Cynara cardunculus extracts, with a content of 34.01% (*w*/*w*) in cynaropicrin were also tested against Gram-positive bacteria, MRSA (Methicillin-Resistant *Staphylococcus aureus*), presenting a Minimum Inhibitory Concentration of 1024 μg/mL that, according to authors, could derive from cynaropicrin content [37]. However, in a another study, Scavo et al. [117] showed that an increase or decrease in the extracts biological activity, may be caused by synergetic or antagonistic effects, where a major antimicrobial activity against gram positive (*Bacillus megaterium, Bacillus subtilis, Bacillus cereus, Rhodococcus fascians, Listeria innocua, Staphylococcus Aureus*) and gram negative bacterial species (*Xanthomonas perforans, Pseudomonas syringae pv. tomato, Escherichia coli, Salmonella enterica, Pseudomonas fluorescens*) was obtained by ethanolic extracts, followed by methanolic and aqueous ones. According to this study, the amount of cynaropicrin was not directly correlated with a higher antibacterial activity indicating that this research theme needs to be better exploited, in order to define the context of cynaropicrin antimicrobial activity.

Considering the above referred biological potential, higher cynaropicrin extract content is of high interest and that could be achieved considering new extraction solvents and methodologies (Table 2.4).

Table 2.4. Cynaropicrin extraction yields from *Cynara cardunculus* leaves obtained for different extraction solvents and methodologies, during the last 10 years (2010-2019). DCM - dichloromethane; EtOH - ethanol; and MeOH - methanol; Et_2O - diethyl ether; DES - deep eutectic solvent; IL - ionic liquid aqueous solution; DW - dry height.

Solvent	Extraction yield (mg/g DW)	Extraction methodology	Extraction conditions (S/L; T (ºC); extraction time)	Ref.
H_2O	13.7*	Immersion	1/10; 20 ºC; 72 h	[118]
MeOH (70%)	23.1*			
EtOH (80%)	17.2*			
DCM	40.3	Soxhlet Extraction	1/16; 40 ºC; 7 h	[122]
Ethyl acetate	55.3	Ultrasound Assisted Extraction	1/16; 40 ºC; 5 min	[122]
EtOH	55.0			
EtOH (40%)	41.4			
H2O	23.76			
DES aqueous solution (30%)	62.0	Batch Extraction with stirring	1/30; 25 ºC; 1 h	[116]
DCM	86.5	Soxhlet Extraction	1/30; 7 h	[115, 116]
IL aqueous solution	64.7	Batch Extraction with stirring	1/20; 25 ºC; 1 h	[115]
Water	6.76			
Acetone	3.46			
n-hexane	0.37			
Water	54	Immersion	1/10; 20 ºC; 72 h	[240]
MeOH (70%)	107			
EtOH (80%)	158			

*cynaropicrin concentration (mg/L)

2.5.1. Extraction solvents for Cynaropicrin

A wide range of solvents with different polarities has been applied for conventional cynaropicrin extraction from *Cynara cardunculus*. As observed for the global SL extraction (described in section 2.2), pure water and methanol did not seem to be suitable extraction solvents in terms of extraction yield, compared to ethanol (Table 2.4), although a higher cynaropicrin concentration was achieved with methanol (23.1 mg/L) [117, 240].

Applied for the first time for cynaropicrin extraction from *Cynara cardunculus* leaves, ionic liquids (IL) and deep eutectic solvents (DES) aqueous solutions were recently used under controlled stirring and temperature and a comparison was made using n-hexane, acetone and water under the same extraction conditions and dichloromethane by soxhlet extraction [115, 116]. Aqueous solution of the IL 1-alkyl-3-methylimidazolium chloride and DES composed by quaternary ammonium salts and organic acids, showed to be efficient upon cynaropicrin extraction from *Cynara cardunculus* leaves, with an extraction yield of 64.7 and 62.0 mg/g DW, respectively. Those extraction yields were higher than the ones obtained with pure water or organic solvents under the same conditions, with final cynaropicrin recovery from IL and DES of 65 and 73.6%, respectively. Although the application of these extraction solvents did not achieve higher extraction yields compared to DCM in Soxhlet extraction (86.5 mg/g DW), both presented a high potential as solvents for cynaropicrin extraction from *Cynara cardunculus* leaves.

2.5.2. Extraction methodology for Cynaropicrin

Recently, Brás et al [122] compared conventional (Batch and Soxhlet) and non-conventional (UAE, MAE and PLE) extraction methodologies combined with different extraction solvents (ethanol, ethanol/water, ethyl acetate, water and dichloromethane). The different methodologies studied revealed that combination of ethanol and UAE was the one that presented higher extraction yield (55.00 ± 2.92 mg/g DW), and low energy cost (0.027 kWh/$g_{cynaropicrin}$), for the same extraction temperature and solvent/biomass ratio, 16/1 mL/g.

2.5.3. Extraction solvent and methodology effect upon extract biological activity

Extraction methods as well as extraction solvents must allow higher extraction yields of the compounds of interest, and not modify the compounds chemical structure, in order to not modify their final biological activity.

Solvent selection for the extraction of allelochemicals from *Cynara cardunculus* var. altilis leaves allowed the understanding that application of ethanol as extraction solvent allowed the highest extraction yield, among all extraction solvents under study (ethanol, ethyl acetate, methanol and water) [118]. Ethanolic (between 100 and 93%), as well as ethyl acetate-derived extracts from leaves reveal to be the most active ones, on a wheat coleoptile bioassay and weed germination and growth. The authors demonstrated that ethanol presented the highest extraction yield, however, in terms of biological activity, ethyl acetate extracts presented a slightly higher activity [118].

2.6. Conclusions

Besides their high manufacturing cost, synthetic compounds start to be insufficient to respond to specific human health needs, as well to other biological and biotechnological applications. The extraction of compounds that can be find in Nature, namely in plants, represents an actual broad research subject with high relevance. With the main difficulty associated to plant matrix and the large diversity of compounds, the success of extraction may require an efficient selection of extraction solvent and methodology.

The research community has observed during the last decade to a significant effort on the development of knowledge regarding extraction methodologies and proper solvent selection for several bioactive groups. Although the SL biological potential is known for far, very few efforts have been made to understand the interaction between extraction conditions (solvent and extraction methodology parameters) and SL extraction yield. Mainly applied for SL extraction, conventional methodologies together with toxic and non-environmentally friend solvents present as main disadvantage a high solvent consumption and be time consuming. Although some authors already started to study new SL extraction approaches, with new extraction solvents, achieving relevant improvements there is still insufficient research regarding a systematic SL extraction optimization. The investment in this research area could lead, in the future, to new concepts for SL economic valorization, such as SL extraction by design, with mixtures of solvents, and extraction

methodologies being applied and adjusted to the final SL goal-application, in the perspective of chemical composition and bioactivity. These approaches imply investment in those research areas in order to increase SL extraction yield by sustainable, less costly and more effective solvents and methods, which also need to assure the final SL extract bioactivity.

With a large number of SL already identified, mostly available from the Asteraceae family, and some of them presenting a proved high biological and economic interest, the main challenge for future SL extraction will be the achievement of an ideal combination of biomass/solvent(s)/method(s)/bioactivity/cost.

3

Effect of extraction methodologies and solvent selection upon cynaropicrin extraction from *Cynara cardunculus* leaves

Published as: Brás, T., Neves, L. A., Crespo, J. G., & Duarte, M. F. (2020). Effect of extraction methodologies and solvent selection upon cynaropicrin extraction from *Cynara cardunculus* leaves. Separation and Purification Technology, 236, 116283. https://doi.org/10.1016/j.seppur.2019.116283

Extraction processes for cynaropicrin present in leaves of Cynara cardunculus L. by, T. Brás, L. A. Neves, M. F. Ricardo, J. P. Crespo, (2019, April 10) EP3466936A1 [Online] Available: https://worldwide.espacenet.com/patent/search/family/059337729/publication/EP3466936A1?q=EP3466936A1

3.1. Abstract

Conventional extraction technologies to recover sesquiterpene lactones, relay on long extraction periods, with high temperatures, and use of organic solvents, often leading to degradation of target compounds. Within the present study, the effect of environmentally friendly, and economically viable methodologies, namely ultrasound assisted extraction, using biocompatible solvents, upon cynaropicrin, a sesquiterpene lactone found in *Cynara cardunculus* leaves was evaluated. Hansen solubility parameters, cynaropicrin extraction yield and energy consumption were evaluated. The ultrasound assisted extraction, using ethanol, showed to be the best cynaropicrin extraction methodology, leading to a reduction of 99% of extraction time (7 h to 5 min), a 30% increase of the cynaropicrin extraction yield (40.32 to 55.00 mg/g Dry Weight), and a 97% reduction of energy consumption (1.160 to 0.027 kWh/$g_{cynaropicrin}$), compared to Soxhlet extraction.

The results obtained from this study may be easily transposed to other sesquiterpene lactones extraction processes, with industrial relevance, as an alternative to conventional extraction methodologies.

Keywords: Cynaropicrin-guaianolide-type sesquiterpene lactone; *Cynara cardunculus* leaves extracts; non-conventional extraction methodologies; ultrasound assisted extraction; Hansen solubility parameters

3.2. Introduction

Extraction and separation processes used to recover bioactive compounds need, in general, long working periods, involving exhausting procedures using non biocompatible organic solvents and high energy consumption [226, 227]. To overcome these limitations, new extraction methodologies, referred as non-conventional techniques, are being introduced providing shorter extraction periods, and low energy expenditure and reduce solvents' consumption [225-228]. Ultrasound assisted extraction (UAE), with the main mechanism of action caused by cavitation, has the advantage of low temperature compounds extraction, enhancing both extraction yield and high compounds purity [226]. Microwave assisted extraction (MAE) uses electromagnetic radiation, promoting the increase of thermal energy, potentiating the extraction efficiency, being able to extract both volatile and non-volatile compounds [227]. More recently, pressurized liquid extraction (PLE) has been applied for natural compounds extraction, offering advantages in respect to solvent consumption, extraction yields, extraction time and reproducibility [228]. Besides the non-conventional extraction methodologies referred above, studies have also been carried out using pulsed electric field assisted extraction, enzymatic digestion, supercritical fluids and, more recently, using ionic liquids and deep eutectic solvents [115, 119, 226, 228, 241].

Sesquiterpene lactones (SL) are one of the most prevalent and biologically significant group of secondary metabolites found across the plant kingdom comprising a large group of over 5000 known compounds [16]. Procedures for SL extraction, have been mainly focused on their isolation and identification, after maceration using a large spectrum of solvents, such as methanol and n-hexane, a limitation for industrial applications [29, 159, 242-244]. Recent studies have shown the benefits of using deep eutectic and ionic liquids as extraction solvents comparatively to dichloromethane [115, 119].

Cynaropicrin, a guaianolide SL identified and extracted from *Cynara cardunculus* var. *altilis* (cardoon) leaves, is an interesting compound due to its large availability on cardoon leaves and its great biological potential, as anti-tumoral, anti feedant and anti-inflammatory agent [18, 22, 25, 26, 29, 34, 245, 246].

In this work, different extraction methodologies and different extraction solvents, with a focus on the use of green solvents, were tested for cynaropicrin extraction from cardoon leaves. For that, conventional methodologies, such as batch solid-liquid (BE) and Soxhlet Extraction (SE), were compared with non-conventional techniques, such as Ultrasound Assisted Extraction (UAE), Microwave Assisted Extraction (MAE) and Pressurized Liquid Extraction (PLE). Different solvents with different polarities, namely dichloromethane, ethanol, water and ethyl acetate were tested.

The screening here presented reveals that UAE using ethanol, could be applied to SL extraction, leading to higher yields, and lower extraction periods, conditions which make feasible the scale-up of the extraction process, for industrial applications.

3.3. Materials and Methods

3.3.1. Reagents and standards

Extraction solvents used were dichloromethane (>95%), provided by Normapur (United Kingdom); ethanol absolute and ethyl acetate (99.9%), both provided by BDh Prolabo (France). Acetonitrile (HPLC grade) was provided by Merck (France) and the standard of cynaropicrin was acquired at Extrasynthese (France).

3.3.2. Raw material

Cynara cardunculus L. (DC) leaves were collected in June 2015 at the Experimental Center of Agriculture School of Instituto Politécnico de Beja, Portugal and preserved at -80 ºC. Before extraction, samples were freeze-dried in a freeze-drier (Scanvac coolsafe, Labogene, Denmark). Freeze-dried leaves, with moisture content below the detection limit of 1%, were grounded using a domestic mixed grinder (Moulinex) in order to obtain a powder with particles presenting a size lower than 500 µm.

3.3.3. Extraction

Cynaropicrin was extracted following four different methods in order to understand their effect upon cynaropicrin final extracts concentration. The extraction methods used were solid-liquid Batch Extraction (BE), Ultrasound Assisted Extraction (UAE), Microwave Assisted Extraction (MAE) and Pressurized Liquid Extraction (PLE). The extraction solvents were ethanol, water, ethyl acetate and ethanol/water (40/60). Soxhlet Extraction (SE) using dichloromethane as extraction solvent, was also performed, since it was the reference method for cynaropicrin extraction from *Cynara cardunculus* leaves [29].

In order to use the minimum amount of solvent, different liquid/solid (mL/g) ratios were firstly tested and a liquid/solid ratio of 16/1 mL/g was selected, representing the best compromise between the amount of solvent and the easiness of mixing. The liquid/solid ratio and temperature (40 ºC) were kept constant for all extraction methodologies, for a proper methods comparison. Exception was made for MAE, where temperature was not controlled due to intrinsic solvent heating. Extraction methodologies are individually described below.

3.3.3.1. Soxhlet Extraction (SE)

SE was performed as described elsewhere [29]. Briefly, 5 g of dry weight (DW) *Cynara cardunculus* leaves were Soxhlet extracted with dichloromethane for 7 h. The solvent was evaporated at low pressure on a rotary evaporator (Hei-VAP Advantage, Heidolph, Germany).

3.3.3.2. Batch extraction (BE)

BE was performed at a stirring rate of 900 rpm and an extraction time of 1 h. After extraction, extracts were filtered under vacuum with a glass filter G4 (nominal maximum pore size 10-16 μm). The final supernatant was collected, organic solvents were vacuum-evaporated in a rotary evaporator (Hei-VAP Advantage, Heidolph, Germany), and water freeze-dried in a freeze-drier (Scanvac coolsafe, Labogene, Denmark).

3.3.3.3. Microwave Assisted Extraction (MAE)

MAE was performed on a microwave digestor (Ethos Easy, Milestone Srl, Italy) with a microwave power of 1000 W during 15 min. Determination of absorbed energy, could be determined by the calorimetric method, with main assumption that all absorbed energy is transformed to heat [247] with energy absorbed calculated as given by equation 3.1,

$$E = mCp\ \frac{dT}{dt} \qquad \text{(equation 3.1)}$$

where E corresponds to the microwave energy absorbed (J/min), C_p is the heating capacity of the mixture (solvent + sample) (J/g.K) and $\frac{dT}{dt}$ corresponds to the rate of temperature rise. Although the microwave equipment used for the experiments referred, is equipped with a temperature indicator, no accurate record was possible. Besides that, the heating capacity of the mixture (solvent + sample) was not available, and therefore it was not possible for the authors to calculate it. After cooling to room temperature, extracts were vacuum filtered with a glass filter G4 (nominal maximum pore size 10-16 μm). The final supernatant was collected, organic solvents were vacuum-evaporated in a rotary evaporator (Hei-VAP Advantage, Heidolph, Germany), and water freeze-dried in a freeze-drier (Scanvac coolsafe, Labogene, Denmark).

3.3.3.4. Pressurized Liquid Extraction (PLE)

PLE was performed using an accelerated solvent extractor (ASE 100, Dionex, ThermoScientific, USA). This process was accomplished in a single static cycle during 5 min, with a self-temperature control equipment and with a maximum pressure of 10.77 kPa. Due to equipment limitations, pure water was not tested. The final extract was collected, organic solvents were vacuum-evaporated in a rotary evaporator (Hei-VAP Advantage, Heidolph, Germany), and water freeze-dried in a freeze-drier (Scanvac coolsafe, Labogene, Denmark).

3.3.3.5. Ultrasound Assisted Extraction (UAE)

UAE studies were performed using a UAE probe device (Bandelin HD3200, VS70T probe, Germany), 20 kHz, using an extraction time of 5 min and amplitude of 15% (12.15 μm). Determination of absorbed power by calorimetric method [247] as described for microwave assisted extraction, was not possible, due to the lack of an accurate temperature record. The extraction vessel temperature was measured by a temperature indicator and controlled by an external cooling bath. After extraction, extracts were vacuum filtered with a glass filter G4 (nominal

maximum pore size 10-16 µm). The final supernatant was collected, organic solvents were vacuum-evaporated in a rotary evaporator (Hei-VAP Advantage, Heidolph, Germany), and water freeze-dried in a freeze-drier (Scanvac coolsafe, Labogene, Denmark).

3.3.4. Cynaropicrin quantification by High Pressure Liquid Chromatography (HPLC)

Cynaropicrin was quantified by HPLC. A Dionex Ultimate 3000 system (Thermo Scientific, USA), equipped with a Diode Array detector DAD-3000(Thermo Scientific, USA) was used. A Kinetex F5 2.6µ (4.6 x 150 mm) column, from Phenomenex (USA), was used at 30 ºC, with water:EtOH (75:25) as mobile phase, at a flow rate of 0.5 mL/min. All samples were pre-filtered with 0.22 µm pore size membrane filters (Pall, USA).

3.3.5. Energy consumption

Energy consumption for each extraction methodology was measured using a digital plug-in power meter (Intertek, USA), 230V, 16A, accuracy class of 2%.

3.3.6. Computational methods

Hansen Solubility Parameters (HSP) analysis was based on the concept that the total cohesive energy density is approximated to the sum of the energy densities required to overcome atomic dispersion forces (δ_d^2), molecular polar forces arising from dipole moments (δ_p^2) and hydrogen-bonds between molecules (δ_h^2), as given by equation 2:

$$\delta_{total}^2=\delta_d^2+\delta_p^2+\delta_h^2 \quad \text{(equation 3.2)}$$

Where δ_{total} is the Hansen total solubility parameter, which consists in three items: dispersion (δ_d), polar (δ_p), and hydrogen-bonding (δ_h).

The HSP values were calculated using a HSPiP software version 5.0.04. Cynaropicrin HSP parameters were evaluated by the Yamamoto-Molecular Break (Y-MB) method, which can break Simplified Molecular Input Line Entry Syntax (SMILES) into the corresponding functional groups and then estimate its HSP. This method was included in the HSPiP software in order to facilitate the direct HSP calculation.

The chemical structure of cynaropicrin was transformed into its SMILES using an online database [248].

3.3.7. Statistical analysis

All experimental results were performed at least in triplicate (n = 3) and the data are expressed as means ± standard deviation. Cynaropicrin data concentration (mg/g DW) within the different solvents and different extraction methods used was analyzed by Prism version 7.00 for Windows (GraphPad Software, USA). Where differences did exist, the source of the differences at a $p < 0.05$ of significance level was identified by all pairwise multiple comparison procedure. The Tukey's test was used for pairwise comparisons.

3.4. Results and Discussion

3.4.1. Solvents effect upon cynaropicrin extraction yield: approach with Hansen solubility parameters

The extraction solvent to be used represents an important factor that partially determines the efficiency of extraction methodologies and should be selected accordingly to the analyte to be extracted. Different extraction solvents, with diverse physical and chemical properties, were tested for cynaropicrin extraction.

For conventional extraction, such as batch extraction, the solvent affinity towards target compounds is the main factor. The Hansen solubility is a parameter that quantifies the capability of a solvent to solubilize a target compound. As previously mentioned, it is defined by three parameters: δ_D (dispersion forces), δ_P (permanent dipole-permanent dipole forces) and δ_H (hydrogen bonding) [249]. Table 3.1, presents the HSP parameters for the solvents under study and their relative energy difference (RED) number, calculated with respect to cynaropicrin extraction.

Table 3.1. HSP and RED for Ethanol (EtOH); Ethyl acetate; EtOH/water (40/60), Water, Dichloromethane (DCM). RED calculation was performed considering cynaropicrin as target compound.

	HSP				
	δ_d (Mpa$^{1/2}$)	δ_p (Mpa$^{1/2}$)	δ_h (Mpa$^{1/2}$)	δ_{total} (Mpa$^{1/2}$)	RED
EtOH	15.8	8.8	19.4	26.5	1.44
Ethyl acetate	15.8	5.3	7.2	18.2	0.36
EtOH/Water (40/60)	15.6	13.1	33.1	38.9	2.90
Water	15.5	16.0	42.3	47.8	3.89
DCM	17.0	7.3	7.1	19.8	0.49
Cynaropicrin	16.7	2.3	7.2	18.3	-

Analysis of RED values provides information about the strength of solvent-solute interactions. A RED value lower than 1, represents a solvent with a character similar to the solute and consequently with a high ability to dissolve it, while RED values between 1 and 3, correspond to solvents with a moderate solubility power. RED values higher than 3 represent solvents with a poor solubility affinity towards the solute under study [249]. According to the RED values calculated, cynaropicrin should present a high solubility in ethyl acetate and DCM (RED<1), moderate solubility in ethanol and in the mixture of ethanol/water (1<RED<3), and poor solubility in water.

The results obtained for the cynaropicrin extraction yield when using conventional extraction methods, batch and soxhlet extractions, are in agreement with the RED analysis, for ethyl acetate, DCM and water (37.48 ± 2.19 mg/g DW, 40.32 ± 1.13 mg/g DW and 13.60±1.13 mg/g DW respectively). Exception was found for ethanol, which presents the highest cynaropicrin extraction

yield (56.90 ± 1.47 mg/g DW), a result not expected according to the HSP approach. Based on the literature, Bundeesomchok et al. [250] and Filly et al. [251], previously reported that the Hansen solubility in Practice (HSPiP) and the Yamamoto-Molecular Break (Y-MB) applications might present some limitations on the prediction of solubility when large solvent molecules were used (e.g. DCM and aromas) for α-mangostin extraction, which could explain the deviation on the prediction of cynaropcrin solubility with ethanol, obtained in our study. Moreover, the natural highly complex plant matrix could induce some cynaropicrin extraction enhancement, which may not be completely predicted by HSP parameters.

3.4.2. Influence of extraction methodologies: non-conventional extraction methodologies

Microwave Assisted Extraction (MAE)

MAE is based on the solubility of the analyte within the solvent, interaction between the solvent and the plant matrix, and microwave absorbing solvent properties [228]. The enhancement of product recovery by microwave is generally attributed to its localized heating effect, which occurs due to the dipole rotation of the solvent in the microwave field increasing the solubility of the compound of interest, and their diffusivity in the extraction medium [252]. The success of MAE is mainly attributed to its high selectivity that favors polar molecules, and two other parameters: the dielectric constant (ε') and the dissipation factor (tan β) [253]. The first one allows to relate the solvent ability to absorb microwave energy, while dissipation factor measures the efficiency with which different solvents heat up under microwave radiation.

The dissipation factor is defined as:

$$\tan\beta = \frac{\varepsilon''}{\varepsilon'} \qquad \text{(equation 3.3)}$$

Where, ε'' is the dielectric loss factor, and ε' corresponds to the dielectric constant (F/m). A solvent that heats up rapidly under microwave radiation typically has high dielectric constant and a low dissipation factor.

From the solvents under study for cynaropicrin MAE, water presented the lowest cynaropicrin extraction yield (1.47 ± 0.16 mg/g DW) and ethyl acetate the highest value (38.41±1.61 mg/g DW). When comparing the dielectric constants of these two solvents (Table 3.2), the first one presents a higher value comparatively to the dielectric constant for ethyl acetate. Regarding to the dissipation factor, no value is available for ethyl acetate in literature, since it is considered a microwave-transparent solvent, which means that it absorbs a very low amount of microwave energy. For microwave-transparent solvents, the behavior observed may be explained by the "broken cell-wall theory". According to this, in the presence of microwave-transparent solvents, plant material is the only one that absorbs microwave energy [254] . Since plant cellular structure possesses water and can absorb microwave energy, the absorbed energy promotes a very quick increase of local temperature inside the cells, which may result eventually on the break of cell

walls and the release of compounds to the solvent [108, 254]. When ethyl acetate is used as an extraction solvent and considering the "broken cell-wall theory", cynaropicrin is released to the solvent, which could explain the high extraction results obtained for this extraction solvent when compared to the others under study.

Table 3.2. Dielectric constant and dissipation factor for ethanol, ethyl acetate and Water.

Solvent	ε' at 20 ºC	tan β (2.45GHz)
Ethanol [252]	24.3	0.2286
Ethyl acetate [228]	6.02	n/a
Water [252]	78.4	0.15

n/a – not available in literature

On the other hand, water presents a low dissipation factor, indicating that it can absorb the microwave energy but not dissipate it significantly, which is described by several authors as "superheating effect" [252, 254]. In the superheating effect, intense energy absorption may cause degradation of the compounds to be extracted, what could explain the low cynaropicrin extraction yield comparing to the other solvents under study and also when comparing to the batch extraction.

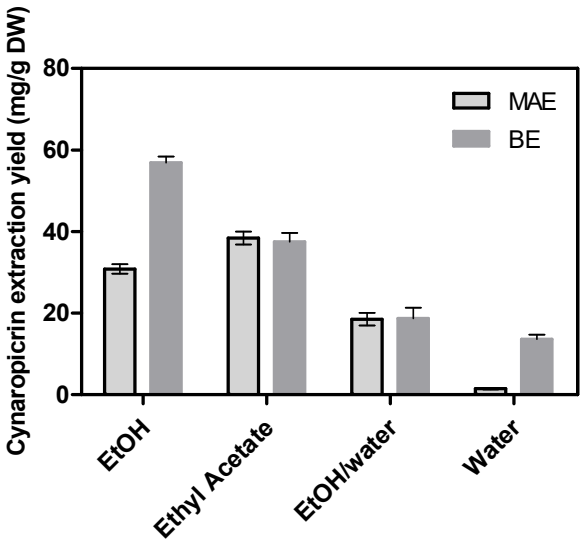


Figure 3.1. Cynaropicrin extraction yield (mg/gDW) in the extracts obtained by Microwave Assisted Extraction (MAE) and Batch Extraction (BE) using Ethanol (EtOH), Ethyl acetate, EtOH/water mixture and water.

Although ethanol can be considered as an efficient solvent for cynaropicrin extraction in conventional extraction methodologies, the application of ethanol with MAE promoted a decrease on extraction yield, comparatively to BE (Figure 3.1). Ethanol presents a moderate dielectric constant value, the heat dissipation to the surrounding environment is not the most efficient one, causing a local temperature increase. Previously, Alfaro et al [22] demonstrated, during ginger extraction with pure ethanol, that the absorption of microwaves by ethanol is significant, leading to system heating by at least two competing mechanisms: direct heating from ethanol and microwaves interaction, and heating from transport of excess heat. As a consequence of these

two mechanisms, an increase of temperature up to 80 ºC may be reached which, in the case of thermolabile compounds such as cynaropicrin, may result in thermal degradation, explaining the MAE lower cynaropicrin extraction yield, when compared to BE methodology (30.82±1.15 and 56.90±1.47 mg/g DW, respectively).

Despite this, the combination of ethanol and water as extractions solvents, in a proportion of 40/60 (v/v) seems to neutralize the "superheating effect" of water and ethanol, with no statistical difference being observed between cynaropicrin extraction yield (mg/g DW) for MAE and BE, with extraction efficiency being mainly controlled by diffusion effects.

Pressurized liquid extraction (PLE)

PLE implies application of high pressure in order to keep the extraction solvent in liquid state, beyond its normal boiling point (at atmospheric pressure). The mechanism of PLE is based on the solvent affinity with the analyte, with high temperature and pressure. Since the temperature used in this work (40 °C) is below the boiling point (at atmospheric pressure) for the solvents under study, it is suggested that besides analyte solubility, pressure will be also a dominant effect. In addition, when combined with high temperatures, high pressure facilitates the extraction process by increasing both solubility and mass transfer and, also decreasing the viscosity and surface tension of the solvents [226, 233]. This may also promote cell wall disruption which will increase mass transfer across cells [232]. In fact, this was observed when ethyl acetate and the mixture ethanol/water (40/60 v/v) was used, with an increase of 48% and 189% on cynaropicrin extraction yield, respectively (Figure 2). The same behavior was not observed when ethanol was used as extraction solvent with a decrease on cynaropicrin extraction yield being observed, when using PLE (47.94 ± 3.60 mg/g DW) comparatively to BE (56.90 ± 1.47 mg/g DW) (Figure 3.2).

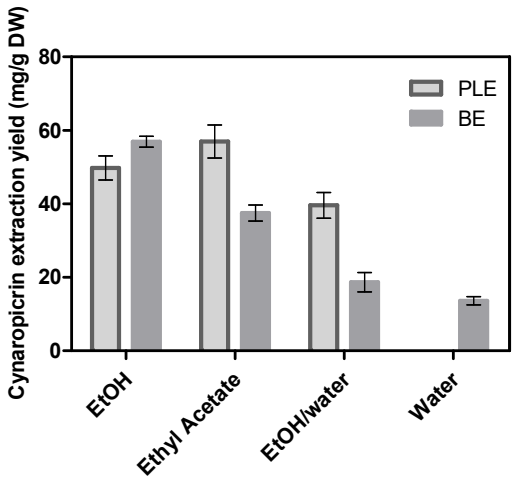


Figure 3.2. Cynaropicrin extraction yield (mg/g DW) in the extracts obtained by Pressurized Liquid Extraction (PLE) and Batch Extraction (BE) using different solvents: Ethanol (EtOH), Ethyl acetate, EtOH/water mixture and Water.

Ultrasound assisted extraction (UAE)

The efficiency of UAE is mainly attributed to mechanical, cavitation and thermal effects, that can provide the disruption of cell walls, promote particle size reduction and enhance mass transfer [228]. This enhancement promotes an increase on extraction yield and it could be observed when ethyl acetate and water were applied, as extraction solvents, with a cynaropicrin extraction yield increase, respectively, of 60 and 26% (Figure 3.3). For the mixture of ethanol/water, an increase on cynaropicrin extraction yield of 179% was achieved, indicating that besides the disruption on cell walls and diffusion mechanisms, other solvent-solute interactions may be potentiated during UAE. Grieser et al [255] suggested that cavitation bubbles formed during the application of ultrasound energy to polar liquids induce the formation of gas-liquid interfaces that facilitate the interaction with hydrophobic moieties of the molecules, making possible to extract non-polar components, such as cynaropicrin, into hydrophilic aqueous extraction media.

This could explain the reason why there is a high increase on cynaropicrin extraction yield for the mixture ethanol/water. Moreover, the presence of water increases the effectiveness of plant tissues swelling, which improves the surface area for solute-solvent contact [256].

Shotipruk et al. [257] showed that menthol extraction by UAE, leaded to surface peeling and particle breakdown, potentiating mass transfer. The authors explained that there were two mechanisms involved: i) diffusion of the target compound through the cuticle of peppermint glandular trichomes; and ii) exudation of the product from broken and damaged trichomes. Eljounaidi et al. [258] revealed that cynaropicrin from globe artichoke predominantly accumulates in glandular trichomes and not in the apoplastic cavity fluids. Therefore, and according to the hypothesis of Shotipruk et al. [257], our UAE results might be explained by the improvement of solvent release, and mass transfer increase, potentiating cynaropicrin diffusion and exudation from broken trichomes.

A comparative analysis for the different extraction methodologies, and solvents under study, shows that UAE with ethanol or with ethyl acetate as extraction solvents (55.00±2.92 mg/g DW and 52.57±1.73 mg/g DW, respectively) and PLE with ethyl acetate (56.96±4.52 mg/g DW) presented the best results in terms of cynaropicrin extraction yields (Table 3.3).

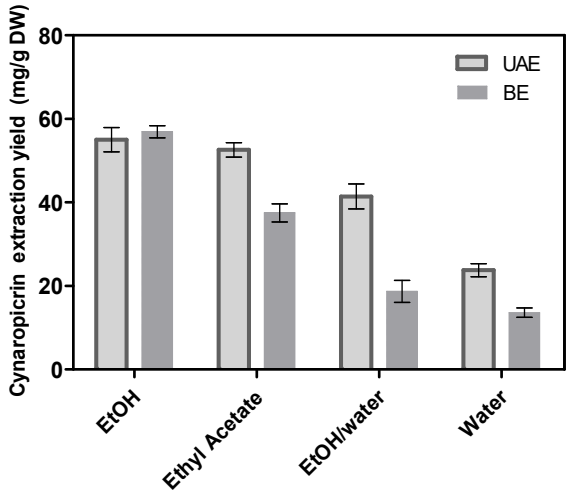


Figure 3.3. Cynaropicrin extraction yield (mg/g DW) in the extracts obtained by Ultrasound Assisted Extraction (UAE) and Batch Extraction (BE) using different solvents: Ethanol (EtOH), Ethyl acetate, EtOH/water mixture and Water.

Table 3.3. Cynaropicrin extraction yield (mg/g DW) for different solvents, ethanol (EtOH), ethyl acetate, EtOH/ water mixture, water and dichloromethane (DCM) and different extraction methods (Soxhlet Extraction - SE; Batch Extraction - BE; Ultrasound Assisted Extraction - UAE; Microwave Assisted Extraction - MAE and Pressurized Liquid Extraction - PLE).

Extraction Solvent	Extraction Method				
	SE	BE	UAE	MAE	PLE
EtOH	-	56.90 ± 1.47^{a}	55.00 ± 2.92^{a}	30.82 ± 1.15^{c}	47.94 ± 3.60^{b}
Ethyl Acetate	-	37.48 ± 2.19^{b}	52.57 ± 1.73^{a}	38.41 ± 1.61^{b}	56.96 ± 4.52^{a}
EtOH/water (40/60)	-	18.69 ± 2.63^{b}	41.42 ± 3.01^{a}	18.51 ± 1.54^{b}	39.59 ± 3.48^{a}
Water	-	13.60 ± 1.13^{b}	23.76 ± 1.56^{a}	1.47 ± 0.16^{c}	-
DCM	40.32 ± 1.13	-	-	-	-

Average±standard deviation estimated from 3 aliquots of 3 extracts.
Values with different superscripts, within the same column, are statistically different ($P < 0.05$)

Taking into account the results obtained for SE (40.32±1.13 mg/g DW), the approach of using less or non-toxic organic solvents with high affinity to cynaropcrin was successful. Moreover, the new extraction methodologies have the main advantage of reducing the extraction time, constituting the best option for cynaropicrin extraction from *Cynara cardunculus* leaves.

3.4.3. Energy consumption

According to previous work from our research team [29], Soxhlet Extraction took 7 h, while for the new tested methodologies, the maximum extraction time was 1 h for BAE, and 5 min for UAE. In perspective of scaling-up the process, energy consumption is of major importance and great economic impact. Therefore, for better comparing the results obtained, the energy consumption/g cynaropicrin extracted was measured for the best solvent for each extraction method and the results are shown below (Table 3.4).

Table 3.4. Energy consumption for Cynaropicrin extraction methodologies under study (Batch Extraction - BE; Ultrasound Assisted Extraction - UAE; Microwave Assisted Extraction - MAE and Pressurized Liquid Extraction - PLE).

Extraction method	Solvent	Energy ($kWh/g_{cynaropicrin}$)
BE	Ethanol	1.160
UAE	Ethanol	0.027
MAE	Ethyl Acetate	0.742
PLE	Ethyl Acetate	0.176

Table 3.4 shows that UAE, using ethanol as solvents, presented by far the lowest energy consumption. When comparing non-conventional extraction techniques with conventional ones, generally non-conventional methodologies present a superior or similar ability to produce extracts rich in cynaropicrin, for the same extraction solvent (Table 3.3). Additionally, non-conventional methodologies require a shorter extraction time, which also translates into lower energy costs.

3.5. Conclusions

Conventional methodologies described in the literature for the extraction of sesquiterpene lactones use non-biocompatible and/or toxic solvents, involving also a high consumption of energy [29, 115, 119, 259]. The study of cynaropicrin extraction, a sesquiterpene lactone present in *Cynara cardunculus* leaves, is of high interest, due to its broad range of biological properties [18, 22, 25, 26, 34, 245, 246]. The results achieved in this work demonstrate that the Hansen solubility parameters are of major importance for the prediction of analyte solubility in conventional extraction methodologies, but with some limitations that may be attributed to the complexity of the plant matrix. Ethanol and ethyl acetate showed to be the best extraction solvents, when using BE, UAE and PLE as extraction methodologies. On the other hand, MAE extraction showed the lowest extraction efficiency mainly due to an overheating effect and consequent degradation of cynaropicrin. BE cannot be considered as an adequate methodology for cynaropicrin extraction due to the high extraction time and energy consumption required.

In this study it was demonstrated that Ultrasound Assisted Extraction, in the presence of ethanol, is a promising technique to obtain cynaropicrin, a guaianolide-type sesquiterpene lactone present in *Cynara cardunculus* leaves.

4

Ultrasound Assisted Extraction of Cynaropicrin from *Cynara cardunculus* leaves: optimization using the Response Surface Methodology and the Effect of Pulse Mode

Published as: Brás, T., Paulino, A. F. C., Neves, L. A., Crespo, J. G., & Duarte, M. F. (2020). Ultrasound assisted extraction of cynaropicrin from *Cynara cardunculus* leaves: Optimization using the response surface methodology and the effect of pulse mode. Industrial Crops and Products, 150, 112395. https://doi.org/10.1016/j.indcrop.2020.112395

4.1. Abstract

In this study, conditions for Ultrasound Assisted Extraction (UAE) of cynaropicrin from *Cynara cardunculus* leaves by assessing the effect of pulsed mode and different duty cycles were optimized. Pulse mode presented a reduction of 45% on energy consumption/ g cynaropicrin extracted, comparatively to continuous mode, and a duty cycle of 25% presented the highest kinetic rate between the duty cycles under study, with a lower number of pulse repetitions necessary to reach a steady state conditions. The extraction methodology was further optimized by response surface methodology (RSM), using a Box-Bhenken design with 3 factors (solid/liquid ratio, amplitude and temperature), and 3 levels for correlation of independent variables with the extraction yield (mg of cynaropicrin/g dry weight) and cynaropicrin concentration (mg cynaropicrin /g extract). Experimental results were fitted to a second order polynomial model, using multiple regression and analysis of variance to determine the fitness of the model. Optimal conditions were found for a solid/liquid ratio of 1/27, amplitude of 67% and temperature of 44 ºC, with a predicted value of 23.99 mg/g DW and 192.23 mg/g extract for extraction yield and cynaropicrin concentration, respectively. The predicted values fit the experimental ones, with a 95% confidence level showing the accuracy of the model. The results obtained suggest the applicability of the UAE optimization methodologies

described, for cynaropicrin extraction from *Cynara cardunculus* leaves, illustrating its potential application on biotechnological and agro food industries.

Keywords: Cynaropicrin; Ultrasound assisted extraction; Pulse mode; Response surface methodology; *Cynara cardunculus*

4.2. Introduction

Cynara cardunculus (cardoon) is a perennial plant, from the Asteraceae family, exhibiting a vigorous growth, with great adaptation to Mediterranean climates. Within the different plant parts, leaves represent about 35.4% dry weight of the total plant [260], and are a great source of natural compounds, with known potential as antioxidant, antibacterial, antitumoral, anti-feedant among others, with high applicability in food and pharmaceutical industries [18, 22, 26, 34, 245, 246, 261, 262]. In an earlier work chemical characterization of lipophilic [29] and phenolic [263] constituents of several parts of *Cynara cardunculus* var. altilis was found, demonstrating that leaves present a high amount of sesquiterpene lactones (SL) (94.5 g/kg dry weight (DW)), with cynaropicrin as the major SL (87.5 g/kg DW) found [29]. Some studies have demonstrated the cynaropicrin biological potential, with positive results being achieved regarding its antitumoral and anti-inflammatory potential, as well its use within a more sustainable agriculture and food industry [117, 118, 264].

The growing interest in plant-derived products prompts continual search for green and economically feasible extraction methodologies. In a recent study from our research group, conventional extraction methodologies were assessed *versus* non-conventional ones, using different extraction methodologies, and their impact upon cynaropicrin extraction efficiency from *Cynara cardunculus* leaves. It was found that Ultrasound Assisted Extraction (UAE), with ethanol as extraction solvent, is the best extraction methodology among the ones studied, leading to a higher yield and a lower energetic cost comparatively to conventional extraction methodologies described for cynaropicrin extraction, such as extraction with mechanical stirring and soxhlet extraction [122].

The efficiency of UAE is mainly attributed to mechanical, cavitation and thermal effects, that can lead to the disruption of plant cell walls, promote particle size reduction and enhance mass transfer [228]. UAE performance may be quantified by several variables, such as solid/liquid (S/L) ratio, temperature and ultrasound amplitude [265-271]. Advantages on the use of pulsed mode instead of continuous processing have been reported, regarding extraction of biologically active compounds, namely: lower energy input, better temperature control and a decrease of erosion of the ultrasound probe tip [272-274].

To the best of our knowledge, no studies have been carried out in order to optimize the extraction of natural compounds from *Cynara cardunculus* leaves, neither cynaropicrin from other plants. Herein, the influence of the pulsed mode comparatively to continuous mode on cynaropicrin UAE was performed, aiming the achievement of a high cynaropicrin extraction yield, with low energy

expenditure, followed by optimization of the extraction process conditions (S/L ratio, amplitude and temperature) using a Box-Behnken design with Response Surface Methodology (RSM).

The present work aims the development of a methodology that can be used, as a model, for the extraction of natural compounds from *Cynara cardunculus* leaves, and also establish a start point for cynaropicrin extraction from other botanical species.

4.3. Materials and Methods

4.3.1. Reagents and standards

Ethanol absolute ≥ 99.8% was provided by BDh Prolabo (France), Acetonitrile (HPLC grade) was provided by Merck (France), and Cynaropicrin was provided by Extrasynthese (France).

4.3.2. Raw material

Cynara cardunculus L. (DC) leaves were collected in March 2016 at the Experimental Center of Agriculture School of Instituto Politécnico de Beja, Portugal and preserved at -80 ºC. Before extraction, samples were freeze-dried in a freeze-drier (Scanvac coolsafe, Labogene, Denmark). Freeze-dried leaves, with moisture content below the detection limit of 1%, were grounded using a domestic mixed grinder (Moulinex, France), in order to obtain a powder constituted by particles with a size lower than 500 µm. After gridding, the freeze-dried powder was stored under vacuum, at room temperature till extraction.

4.3.3. Extraction

UAE experiments were performed for two different studies: a) evaluation of pulse mode operation versus continuous operation; and b) experimental design using RSM for optimization of the methodology selected. For both studies, an UAE device (Bandelin HD3200, Germany), 20kHz, equipped with a titanium alloy (Ti-Al6-V4) probe with 13mm diameter (Bandelin, Germany) was used. After extraction, the extract was filtered in a glass filter funnel, with porosity G4 and 10-16 µm maximum nominal pore size. Ethanol was vacuum evaporated at 35 ºC, in a rotary evaporator (Hei-VAP Advantage, Heidolph, Germany) and the dried extracts were kept at 4 ºC for future analysis.

4.3.3.1. Effect of the pulse mode operation

According to the results previously obtained by our research group [122], considering the following optimization by RSM and extraction limitations such as: lowest extraction mixing for S/L higher than 1/16; equipment limitations under 15% ultrasonic amplitude; and the possibility of extract degradation at high temperatures, a solid/liquid ratio of 1/50 g/mL, temperature of 30 ºC and an ultrasonic amplitude of 15% (12.15 µm) were selected as extraction parameters for pulse effect study. Temperature was controlled using an external in-house developed bath. The UAE equipment was operated in a pulse mode, with different duty cycles (25%, 50% and 83.3%). For each duty cycle, 15, 45, 90, 135, 180, 270 and 360 number of pulse repetitions were tested. The duty cycle was calculated as,

$$duty\ cycle\ (\%) = \frac{t_{ON}}{t_{ON}+t_{OFF}} \times 100 \quad \text{(equation 4.1)}$$

where, t_{ON} is the duration time of pulse (s), and t_{OFF} is the time of pulse interval (s). The number of pulse repetitions (t_{ON} + t_{OFF}) corresponds to the number of cycles during the extraction time. In this work, the extraction time was dependent of the number of pulse repetitions, and varies for each duty cycle.

For a better understanding of the pulse mode effect comparatively with the continuous mode, the same experimental conditions (S/L ratio, amplitude and temperature) were tested continuously during 15 min and samples were collected each 30 s during the first 5 min, and each 2.5 min from 5 till 15 min. Each experiment was performed in triplicate.

Recent studies showed that a second order rate model provides an adequate description of the solid-liquid extraction using the ultrasound methodology [275], where the dissolution rate of cynaropicrin is given by,

$$\frac{dC_t}{dt} = k(C_\infty - C_t)^2 \quad \text{(equation 4.2)}$$

where C_t is the concentration of cynaropicrin in the extraction solvent (mg/mL) at a specific time, C_∞ is the concentration of cynaropicrin at a steady state condition (mg/mL), t is the extraction time (min) and k is the extraction rate constant (mL/(mg.min)). Solving the equation, its linear form can be written as,

$$\frac{t}{C_t} = \left(\frac{1}{kC_\infty^2}\right) + \left(\frac{t}{C_\infty}\right) \quad \text{(equation 4.3)}$$

where plotting t/C_t against t, the interception and the slope values can be used to determine the kinetic rate and the steady state condition concentration, respectively.

4.3.3.2. Response surface methodology (RSM)

In order to study the effect of the solid/liquid ratio (1/20 – 1/50 g/mL), the ultrasonic amplitude (15% (12.15 μm) - 85% (68.85 μm)) and temperature (25 ºC – 65 ºC) on the maximum cynaropicrin extraction yield (mg/g DW) and concentration in the extract (mg/g extract), a RSM was used with the Box-Behnken design being selected for the correlation between independent and response variables. For statistical calculation, variables were coded at three levels. A total of 17 experiments including 5 central points were generated with random combination of independent variables. The correlation between independent, and dependent variables was calculated through a second-order polynomial mathematical equation [276] given by:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>1}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad \text{(equation 4.4)}$$

Where Y is the response variable (cynaropicrin extraction yield expressed as mg/g DW, and cynaropicrin concentration expressed in mg/g extract), X_i and X_j define the independent variables, b_0 is the constant coefficient, b_i is the linear effect coefficient, b_{ij} is the interactive effect coefficient, b_{ii} is the quadratic effect coefficient and n is the number of variables. The analysis of the

experimental design and regression analysis was performed using the Design Expert 10.0.7.0 statistical software (Stat-Ease, Inc, Minneapolis, USA). The predicted model significance was evaluated by analysis of variance (ANOVA). The adequacy of the model was evaluated by accounting for the coefficient of determination (R^2), adjusted determination coefficient (R^2_{adj}), coefficient of variance (CV) (%) and signal to noise ratio. A response surface plot was used for evaluation between response and independent variables. The significance of all terms in the polynomial equation was considered statistically different when $p < 0.05$.

4.3.4. Analytical methods: High Pressurized Liquid Chromatography (HPLC)

Cynaropicrin concentration was quantified by HPLC. A Dionex Ultimate 3000 system (Thermo Scientific, USA) equipped with a Diode Array detector was used. A Kinetex F5 2.6 µm (4.6 x 150 mm) column (Phenomenex, USA) was employed at 30 ºC, using as mobile phase a mixture of water and acetonitrile (75:25 (v/v)) with a flow rate of 0.5 mL/min. All samples were pre-filtered with 0.22 µm pore size membrane filters (Pall, USA). Cynaropicrin concentration was determined by the calibration curve (0.05-0.60 mg/mL).

4.4. Results and discussion

4.4.1. Effect of pulse ultrasound assisted extraction

In order to understand the effect of Pulsed Ultrasound Assisted Extraction (PUAE) upon cynaropicrin extraction from *Cynara cardunculus* leaves, cynaropicrin extraction yield was quantified under 3 duty cycles were tested (25%, 50% and 83.3%) and table 4.1, are represented the corresponding extraction times for each duty cycle. For comparison, continuous mode experiments were performed until steady state condition was achieved, measuring the energy consumption.

Table 4.1. Individual and total extraction time (min), for each duty cycle tested (25, 50 and 83.3%) and different number of pulse repetitions, for cynaropicrin extraction from *Cynara cardunculus* leaves on Pulsed Ultrasound Assisted Extraction (PUAE).

Number of pulse repetitions	Extraction time (min)		
	Duty cycle		
	83.3%	50%	25%
15	1.5	2.5	5
45	4.5	7.5	15
90	9	15	30
135	13.5	22.5	45
180	18	30	60
270	27	45	90
360	36	60	120

Table 4.1 shows the results obtained for the 3 duty cycles under study: 25, 50 and 83.3%. For the same number of pulse repetitions, a high duty cycle lead to a lower extraction yield, in comparison

with the lower one. Moreover, the maximum extraction yield is reached after a lower number of pulse repetitions for a duty cycle of 25% (90 pulse repetitions) being the extraction rate higher, as proven by the kinetic rates calculated according to equation 3 (0.765 min^{-1}, 0.133 min^{-1} and 0.104 min^{-1} for 25%, 50% and 83.3%, respectively). A shorter interval for a certain number of pulse repetitions, translates into a shorter extraction time, which may limit mass transfer, as explained by Pan et. al. [277]. After 270 pulse repetitions, for the three duty cycles under study, there was a decrease of the extraction yield, observed for 360 pulse repetitions, which may be related with degradation of the sesquiterpene lactone.

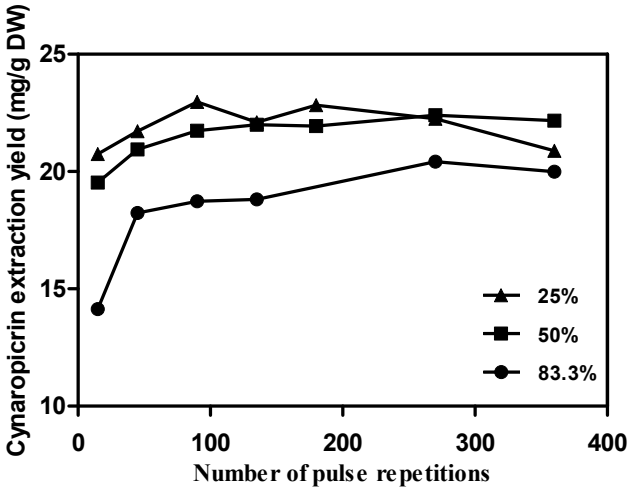


Figure 4.1. PUAE (Pulsed Ultrasound Assisted Extraction) effect upon cynaropicrin extraction yield (mg/g DW) during 15, 45, 90, 135, 180, 270 and 360 number of pulse repetitions for the different duty cycles (25, 50 and 83.3%) at a S/L of 1/50 and 30 ºC of extraction temperature.

When comparing Continuous Ultrasound Assisted Extraction (CUAE) and PUAE, the steady state condition for CUAE is reached after 4 min (Figure 4.2), which compares favorably with the 30 min (90 pulse repetitions) required for a duty cycle of 25% in a PUAE process (Figure 1). During ultrasound extraction, agitation of solvent and solute derived from the implosion of solvents bubbles, promoted by cavitation phenomena can be compared with a mechanical stirring [278]. On CUAE, where ultrasound waves are uninterrupted, the presence of agitation is constant, while for PUAE different periods with no agitation occur. Since agitation, during extraction processes enhances mass transfer, this may explain the higher extraction rate (1.07 min^{-1}) obtained for CUAE when compared with PUAE (0.765 min^{-1}).

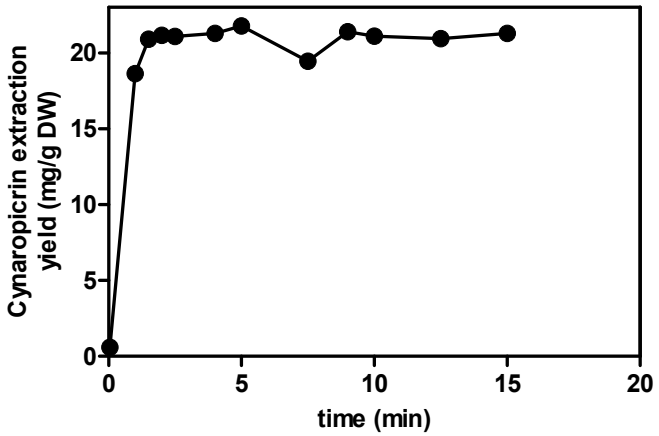


Figure 4.2. Cynaropicrin extraction yield for the Continuous Ultrasound Assisted Extraction (CUAE) process at a S/L of 1/50 and 30 ºC of extraction temperature.

For a better selection of the ultrasonic methodology regarding cynaropicrin extraction from *Cynara cardunculus* leaves, the energy consumption was also taken into account, considering the energy consumption/g of cynaropicrin extracted. The obtained results, shown in Table 4.2, demonstrate that for the same mass of cynaropicrin extracted, the energy consumption is higher for PUAE with a higher duty cycle as well as for CUAE, and lower for PUAE with a lower duty cycle, which was considered to be the best method for extraction, therefore selected for further process optimization.

Table 4.2. Energy consumption for the different pulse intervals under study regarding different extraction methodologies (PUAE (Pulsed Ultrasound Assisted Extraction) with 15, 5 and 1 s of pulse interval and CUAE (Continuous Ultrasound Assisted Extraction) with 4 min, at a S/L of 1/50 and 30 ºC of extraction temperature). UAE_{total} corresponds to total extraction time and UAE_{ON} corresponds to ultrasound "on" time during extraction.

Extraction methodology and Duty cycle	UAE_{total} (min)	UAE_{ON} (min)	Energy consumption (kWh/g cynaropicrin extracted)
PUAE – 25%	30	7.5	0.757
PUAE - 50	45	22.5	1.053
PUAE – 83.3%	27	22.5	1.523
CUAE	4	4	1.378

4.4.2. Response Surface Methodology (RSM)

4.4.2.1. Model fitting

In order to examine and optimize the effect of the main operating parameters (S/L ratio, amplitude and temperature) on the process performance (cynaropicrin extraction yield (mg/g DW) and cynaropicrin concentration (mg/g extract)), a set of 17 experiments with random combinations was performed and the results obtained are shown in Table 4.3.

Table 4.3. Box–Behnken design and observed responses for Cynaropicrin extraction using PUAE for an extraction time of 30min

	Extraction conditions			Responses	
Run	S/L ratio	Amplitude (%)	Temperature (ºC)	Cynaropicrin extraction yield (mg/gDW)	Cynaropicrin concentration (mg/g extract)
1	-1 (1/50)	-1 (15)	0 (45)	24.77	75.80
4	1 (1/20)	1 (85)	0 (45)	24.18	163.68
2	-1 (1/50)	1 (85)	0 (45)	24.07	42.61
3	1 (1/20)	-1 (15)	0 (45)	21.00	56.54
5	-1 (1/50)	0 (50)	-1 (25)	24.12	51.78
6	-1 (1/50)	0 (50)	1 (65)	26.11	48.27
7	1 (1/20)	0 (50)	-1 (25)	21.79	90.29
8	1 (1/20)	0 (50)	1 (65)	24.53	76.64
9	0 (1/35)	-1 (15)	-1 (25)	22.76	57.23
10	0 (1/35)	-1 (15)	1 (65)	23.47	67.00
11	0 (1/35)	1 (85)	-1 (25)	24.43	107.65
12	0 (1/35)	1 (85)	1 (65)	27.07	137.50
13	0 (1/35)	0 (50)	0 (45)	23.60	175.07
14	0 (1/35)	0 (50)	0 (45)	23.07	185.99
15	0 (1/35)	0 (50)	0 (45)	23.02	154.65
16	0 (1/35)	0 (50)	0 (45)	22.20	188.84
17	0 (1/35)	0 (50)	0 (45)	23.40	197.15

The mathematical model to predict the cynaropicrin extraction yield and extract concentration was build up using a multiple regression analysis, and a second order polynomial regression was derived for each response, in order to observe the correlation between dependent and independent variables [276]. The observed data were further fitted to a quadratic function and a variance analysis (ANOVA) was constructed. The obtained results are presented in Tables 4.4 and 4.5, for the extraction yield in mg/g DW (Response 1) and the cynaropicrin concentration expressed in mg/g extract (Response 2), respectively. Factors that showed effect with p-values higher than 0.05 were considered not significant at the 95% confidence level and, consequently, were discarded for the model development. Exception was made for the linear effect of temperature on response 2, because of hierarchy of factors, since the temperature quadratic effect is significant.

Table 4.4. ANOVA results for Cynaropicrin extraction yield, where SS represents the Sum-of-Squares, Df the degree of freedom and MS represents the Mean Squares

Source	SS	Df	MS	F Value	p-value
Model	84.10	9	9.34	15.64	< 0.0001
A-S/L	19.67	1	19.67	32.91	< 0.0001
B-Amplitude	22.14	1	22.14	37.04	< 0.0001
C-Temperature	22.74	1	22.74	38.05	< 0.0001
AB	9.34	1	9.34	15.62	0.0005
BC	2.79	1	2.79	4.67	0.0396
C^2	3.45	1	3.45	5.78	0.0234
R-Squared	0.8321				
Adjusted R-Squared	0.7854				
C.V. %	3.21				
Signal to noise ratio	15.506				

Table 4.5. ANOVA results for Cynaropicrin concentration, where SS represents the Sum-of-Squares, Df the Degree of freedom and MS represents the Mean Squares

Source	SS	Df	MS	F Value	p-value
Model	11900.59	9	13218.74	90.01	< 0.0001
A-S/L	10672.20	1	10672.20	72.67	< 0.0001
B-Amplitude	14238.38	1	14238.38	96.95	< 0.0001
C-Temperature	189.09	1	189.09	1.29	0.2642
AB	14769.80	1	14769.80	100.57	< 0.0001
A^2	42555.97	1	42555.97	289.77	< 0.0001
B^2	14663.06	1	14663.06	99.84	< 0.0001
C^2	32655.38	1	32655.38	222.35	< 0.0001
R-Squared	0.9585				
Adjusted R-Squared	0.9479				
C.V. %	11.93				
Signal to noise ratio	24.98				

Adequacy of models was tested through R-squared, adjusted R-squared, coefficient of variance (C.V.), F-value and p-value [276]. Based on this, with a higher model F-value (15.64 for R1 and 90.01 for R2), with only a 0.01% chance that an F-value this large could occur due to noise, and consequently low p-values (<0.0001), the results obtained suggest an adequacy of the model chosen for the observed data with a 95% confidence level [269, 276]. The capacity of the model to depict the actual correlation among independent variables and responses can be observed by the exhibition of high values of R-squared for both responses (0.8321 and 0.9585). The signal to noise

ratio was high for both responses (15.51 for R1 and 24.98 for R2), which indicates the fitness of the developed models [276].

From the mathematical model, the following second-order polynomial equations (equation 4.5 and 4.6) were obtained in its coded form:

$$Y_1 = 23.60 - 0.96A + 0.99B + 1.00C + 0.93AB + 0.48BC. + 0.72C^2 \quad \text{(equation 4.5)}$$

$$Y_2 = 183.02 + 21.09A + 24.36B - 2.81C + 35.08AB - 61.98A^2 - 36.38B^2 - 54.30C^2 \quad \text{(equation 4.6)}$$

Where, A is the solid/liquid ratio, B is the ultrasonic amplitude (%), C is the temperature (ºC) and Y_1 and Y_2 are the responses expressed for the cynaropicrin extraction yield and concentration, respectively.

The analysis of the second-order polynomial equations is useful for the prediction of the positive or negative effect of a factor, as explained by Heleno et. al. [266]. When a factor has a positive coefficient, the response is higher at the high level, and when a factor has a negative coefficient the response is lower at the high level. Besides that, the absolute value of a coefficient indicates the importance of the corresponding factor.

4.4.2.2. Influence of independent variables upon cynaropicrin extraction yield (mg/g DW)

Regarding the influence of linear and interactive factors, upon cynaropicrin extraction yield from *Cynara cardunculus* leaves, three dimensional surface plots were built (Figures 4.3a, 4.3b and 4.3c).

4.4.2.2.1. Solid/liquid ratio

The effect of solid/liquid ratio on the extraction yield was assessed by varying the solid/liquid ratio in a range of 1/50 to 1/35 g/mL. The analysis of Figures 4.3A and 4.3B shows that a decrease of the liquid phase leads to an extraction yield decrease. An increase of the S/L ratio and a consequent decrease of the liquid phase volume, reduces the contact area among solvent and plant material causing a reduction in dispersion of ultrasound energy in the solvent [268, 276]. This will be translated into a reduction of the rate of solvent transfer into plant cells by diffusion, thus leading to a decrease of the cynaropicrin extraction yield. Besides that, by increasing the liquid phase volume, mass transfer is potentiated due to the higher concentration difference between the solute in the plant matrix and the solvent - higher driving force [269, 276], which may explain the higher cynaropicrin extraction yield at the lowest S/L level.

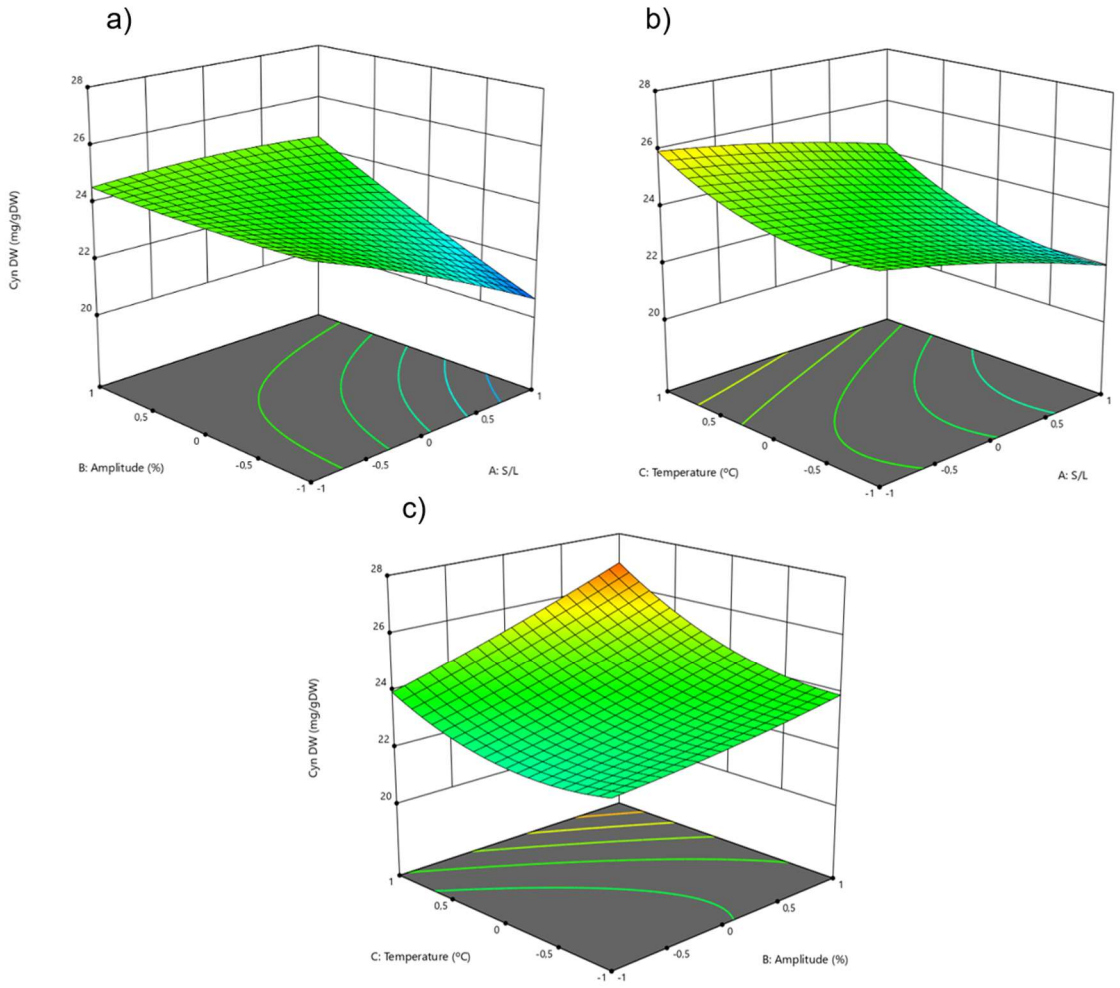


Figure 4.3. Effect of interactive effect of independent variables, S/L ratio (range 1/50 (-1) to 1/20 (+1)) with Amplitude (range 15% (-1) to 85% (+1)) (a), S/L with Temperature (range 25 ºC (-1) to 65 ºC (+1)) (b) and Amplitude with Temperature (c), upon the cynaropicrin extraction yield (mg/gDW)

4.4.2.2.2. Ultrasonic amplitude

The effectiveness of UAE is mainly dependent from the cavitation promoted in the media (solvent and matrix). During the cavitation process, compression and expansion of liquids as well as formation of micro-jets of solvent can be observed, leading to the formation of micropores in the matrix cell, facilitating the access of solvent to the compounds present in intracellular compartments [268, 276, 279]. The cavitation process can be assessed by analyzing the effect of amplitude or the ultrasonic power. Therefore, the ultrasonic amplitude effect upon the cynaropicrin extraction yield was studied by varying it in a range between 15 and 85%, with results being shown in Figures 4.3a and 4.3c. A positive effect can be observed from the multiple regression, on its linear factor (equation 4.5). These results can be confirmed by the analysis of response surface plots, where the extraction yield in mg/g DW is potentiated with the increase of amplitude. An increment of amplitude leads to the formation of more solvent bubbles due to the increase of the cavitation phenomena with stronger liquid jets being created. After a long time of exposure, the swelling of cell wall with the enlargement of pores during the initial stages of cavitation, promotes a collapse of

the cellular wall facilitating a higher transport of solvent through the material matrix, resulting in a higher cynaropicrin extraction rate [276, 280, 281].

4.4.2.2.3. Temperature

Temperature is one of the most important factors in UAE. In this study different temperatures (25 ºC – 65 ºC) and their effect on cynaropicrin extraction yield were studied. According to the results, it was observed that within the temperature range under study, temperature presents a strong positive effect as reflected by equation 5, leading to an increase of the extraction yield.

When analyzing the interactive effect of independent variables upon the cynaropicrin extraction yield (mg/g DW), from Figure 4.3 and equation 4.5, it is possible to observe that the interactive effect of solid/liquid ratio and amplitude presents a positive effect upon the extraction yield, where the highest value was found at the highest amplitude and lowest solid/liquid ratio.

On what concerns the interactive effect of ultrasonic amplitude and temperature, the highest extraction yield was found at the highest temperature and highest amplitude, in the range studied. An increase of ultrasonic amplitude with an increase of extraction temperature, may potentiate the cavitation effect, as previously explained, promoting an increase of the extraction yield, as observed in Figure 4.3. Since the interactive effect of the solid/liquid ratio and temperature did not present a significant effect ($p > 0.05$), no discussion on its interactive effect is made.

4.4.2.3. Influence of independent variables upon cynaropicrin concentration (mg/g extract)

The influence of linear and interactive factors in cynaropicrin concentration ($mg_{cynaropicrin}$ /$g_{extract}$) was analyzed and three-dimensional surface plots were built, as shown in Figure 4.4A, 4.4B and 4.4C.

4.4.2.3.1. Solid/liquid ratio

Concerning the cynaropicrin concentration (mg/g extract), the solid/liquid ratio has the strongest effect , as observed by equation 6, where its quadratic effect presents the highest coefficient, being its effect negative, as explained by Heleno et al. [266]. An increase of the liquid phase volume firstly leads to an increase of the concentration of solute in the extract, but after a maximum value, there was a decrease with the increase of the liquid fraction. This behavior could be expected since as the liquid to solid ratio increases, although the extraction yield also increases, the solute extracted becomes more diluted in the liquid phase.

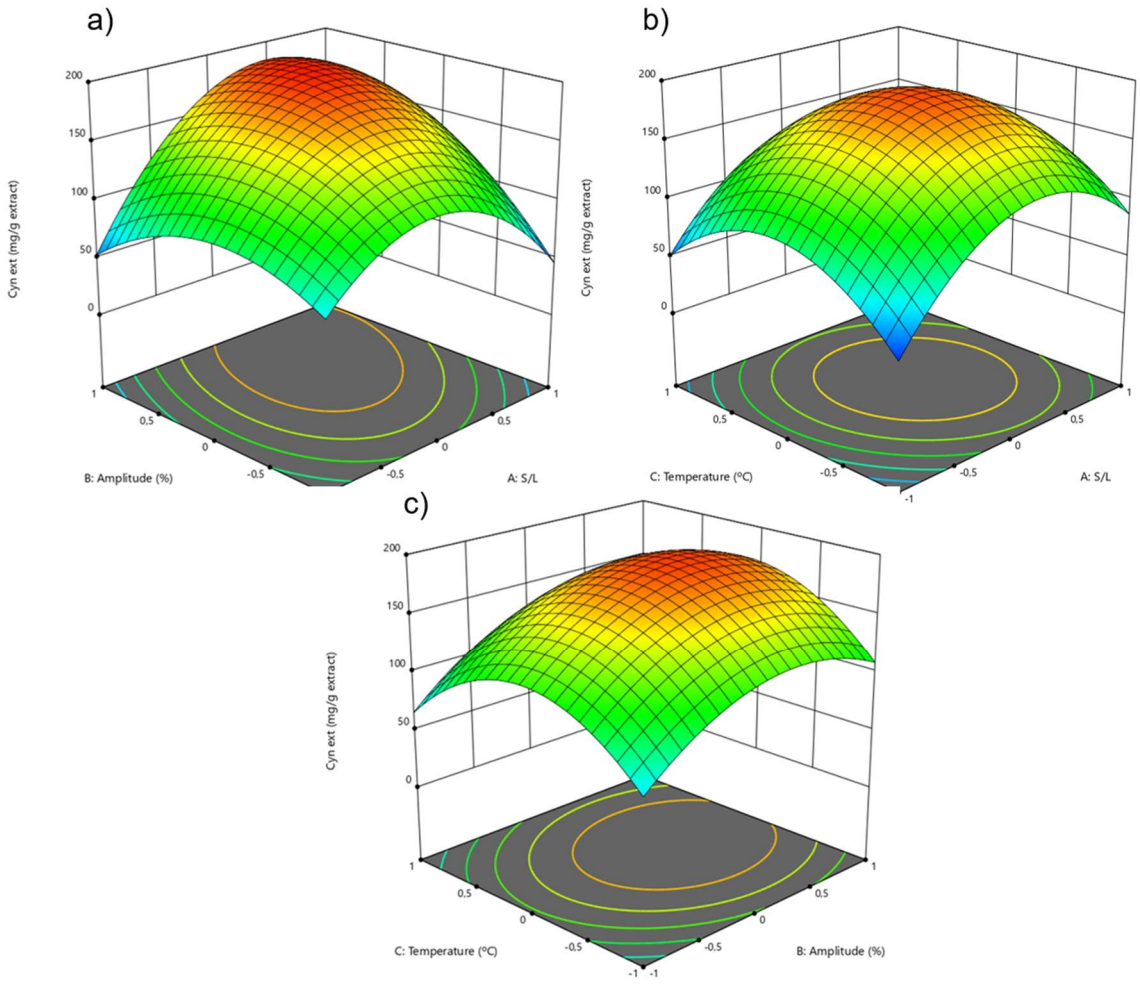


Figure 4.4. Surface plots for the interactive effect of independent variables, S/L ratio (range 1/50 (-1) to 1/20 (+1)) with Amplitude (range 15% (-1) to 85% (+1)) (a), S/L with Temperature (range 25 ºC (-1) to 65 ºC (+1)) (b) and Amplitude with Temperature (c) upon cynaropicrin concentration (mg/g extract).

4.4.2.3.2. Ultrasonic amplitude

The cavitation process, more specifically the liquid compression and expansion of liquids and the formation of jets during the implosion of solvent bubbles, leads to a higher UAE efficiency. The analysis of the ultrasonic amplitude on cynaropicrin concentration, by equation 6, shows a positive linear effect and a negative quadratic effect, with the quadratic effect being higher due to its high coefficient. An increase in amplitude promotes an increase of the cynaropicrin concentration (mg/g extract), explained by the increase of acoustic streaming that forms expansion-compression cycles, that renders the swelling of plant material, enhancing solvent uptake, enlarging the pores of cell wall thus resulting in an increase of efficiency [268, 276]. However, above a certain level of amplitude, a decrease of the cynaropicrin concentration in the extract was observed. Cavitation intensity is strictly compromised if a large number of small bubbles are formed, which depends on the number of compression and expansion cycles generated, which in turns depends on the ultrasonic power and amplitude. Therefore, an increase in amplitude promotes the formation of small bubbles that affect mass transfer due to the reduced transmission of ultrasound energy into

the solvent, and consequently decreases the release of cynaropicrin and may increase the solubility of more accessible molecules present in the *Cynara cardunculus* leaves.

4.4.2.3.3. Temperature

Similarly to the results obtained for cynaropicrin extraction yield, temperature presents a strong effect as reflected by equation 6, but contrarily to the observed on extraction yield, where an increase on temperature promotes an increase of the extraction yield, in which concerns to cynaropicrin concentration, temperature effect is negative (equation 4.6), what may be caused by compound degradation with the temperature increase.

When analyzing the interactive effect of independent variables upon the cynaropicrin concentration (mg/g extract) a maximum is observed for the S/L ratio and amplitude interactive effect with an increase of amplitude, according to Figure 4.4 and equation 4.6. On what concerns the interactive effect of temperature and solid/liquid ratio and amplitude *vs* temperature, due to its non-significant effect, no discussion is made.

4.4.2.4. Optimization and validation

In this study, two responses were studied, the cynaropicrin extraction yield expressed in mg/gDW (X1) and the cynaropicrin concentration expressed in mg/g extract (X2). Optimization was firstly performed taking into account the maximization of the responses in order to obtain an extract with high yield of recovery, and high concentration of the target solute. A numerical optimization method was employed, and the second-order polynomial equations described previously in equations 5 and 6 were solved. The optimal conditions obtained in coded form were: S/L ratio = 0.4272, amplitude = 0.9999 and temperature = 0.5803, which corresponds to a S/L ratio of 1/25.5, an amplitude of 85% and a temperature of 53 ºC, with predicted values of cynaropicrin yield of 25.80 mg/g DW and a concentration in the extract of 169.31 mg/g. Although a simultaneous X1 and X2 optimization could seem to be the most appropriated optimization approach, when looking for the effects on the yield of cynaropicrin extraction mg/g DW, obtained in the present study, the range of results achieved was from 20 to 27 mg/g DW and the range of results obtained for the extract concentration was between 32.89 and 197.15 mg/g extract, which indicate that the independent variables effect is more pronounced in the concentration (mg/g extract) response. Based on this, the optimization procedure was tested to maximize the extract concentration, keeping the cynaropicrin yield in mg/g DW, within the range studied. The results obtained in coded form were: S/L ratio = 0.3068, Amplitude: 0.4849 and Temperature: 0.0407, which corresponds to a S/L ratio of 1/27, amplitude of 67% and temperature of 44 ºC, with a predicted value of 23.99 mg/g DW and 192.23 mg/g extract. From an industrial point of view, the second optimization, where only the concentration of target solute in the extract was maximized, while the extract yield was kept in the range studied, presents economic advantages due to the fact that the optimal determined amplitude and temperature are lower when compared with the ones that assure the maximization of both responses. Lower ultrasonic amplitude as well as lower temperature represent a lower energy expenditure, leading to lower costs.

Triplicate experiments were performed using the optimal conditions selected and the average results for cynaropicrin were a yield of 23.90 ± 0.14 mg/g DW and a concentration of 192.51 ± 6.96 mg/g extract. Comparison between experimental data and predicted values did not show any statistical difference, indicating the accuracy of the model developed and confirming the optimal conditions identified.

4.5. Conclusions

In this work, the effect of pulse mode and independent variables such as S/L ratio, amplitude and temperature, upon cynaropicrin extraction from *Cynara cardunculus* leaves was studied. Cynaropicrin concentration was affected by the duty cycle with a steady state of extraction being achieved with a lower number of pulse repetitions for the duty cycle of 25%. The pulse mode, comparatively with the continuous mode, exhibited a 45% decrease on energy consumption/g of cynaropicrin extracted, with a similar extraction kinetic rate, being selected as the most suitable method of extraction for the RSM optimization. The S/L ratio, amplitude and temperature individual effects on the cynaropicrin extraction yield were evaluated, as well as the interactive effect of S/L ratio and amplitude and the last with temperature. The individual effects on cynaropicrin concentration expressed in mg/g extract were obtained for the S/L ratio and amplitude, as well as the interactive effect of these variables. The optimal conditions obtained for maximization of cynaropicrin concentration in the extract were a S/L ratio of 1/27, an amplitude of 67% and a temperature of 44 ºC. The responses obtained, with optimized conditions, led to a final cynaropicrin content of 192.51 ± 6.96 mg/g, with an extraction yield of 23.90 ± 0.14 mg/g DW, showing no statistical differences with the predicted ones, validating the accuracy of the model developed. The optimization of cynaropicrin extraction from *Cynara cardunculus* leaves, opens perspectives for application of this extraction optimization methodology towards the extraction of bioactive compounds from *Cynara cardunculus* for biotechnological purposes, aiming valorization, as well as the use of this methodology for extraction of cynaropicrin from other botanical sources.

5

Fractionation of *Cynara cardunculus* ethanolic extracts using diananofiltration

Brás, T., Paulino, A. F. C., Neves, L. A., Crespo, J. G., & Duarte, M. F. (2020). Fractionation of *Cynara cardunculus* ethanolic extracts using diananofiltration. Separation and Purification Technology, under review ID SEPPUR-D-20-01874 R1

5.1. Abstract

Cynara cardunculus (cardoon) leaves are a rich source of bioactive compounds, such as cynaropicrin. In this work, Ethanolic Pulsed Ultrasound Assisted Extraction (EtPUAE) derived extracts obtained from cardoon leaves were purified by membrane processing and the different fractions obtained were evaluated for their biological potential.

A DuraMem® 200 membrane was selected for cynaropicrin recovery and removal of carbohydrates, in a diananofiltration mode. Diananofiltration (after 5 diavolumes) followed by nanofiltration allowed for a removal of 93.0% of glucose and 95.6% of fructose, with a cynaropicrin maximum loss of 13.9%. The fractionated extract revealed a higher biological activity, when tested with a BJ5-ta human normal fibroblast cell line. An integrated process is proposed, comprising an association of EtPUAE with a fractionation by diananofiltration, assuring a pay-back period of 4.58 years, assessed through an economic evaluation.

Keywords: Organic solvent nanofiltration; Cynaropicrin; Carbohydrates; Diananofiltration; Biological activity; Economic evaluation

5.2. Introduction

In the concept of economical and biological valorization of natural resources, where plants represent a wide group, chemical analysis of their morphological components may indicate the best way for their valorization. With flowers typically applied as rennet for cheese manufacturing and with a biomass production that can go from 7.8 ton till 20 ton DW/ha, *Cynara cardunculus* (cardoon) research interest has grown in the last years [27, 28, 260, 282]. Aiming for their total exploitation, cardoon has been studied as a potential source of lignocellulosic biomass (stems and whole plant) for oil production (seeds) and as a source of bioactive compounds (leaves and roots), with the latter representing 33.1% to 48.4% regarding the total biomass average weight [27, 28, 283].

Presenting high biological potential, with anti-inflammatory, anti-proliferative and anti-photoaging activities, sesquiterpene lactones can be found in lipophilic extracts from cardoon leaves in amounts that can reach up to 95 mg/g DW, being cynaropicrin the most abundant sesquiterpene lactone found (87 mg/g DW) [29]. Previous studies from our research group, upon cynaropicrin extraction optimization from cardoon leaves, revealed that pulsed ultrasound assisted extraction (PUAE), with ethanol as extraction solvent, allowed for a 30% increase on cynaropicrin extraction yield and a 97% decrease on energy consumption, comparing with Soxhlet extraction [122, 284].

Interactions between constituents of complex natural plan extracts might be of high importance, defining the extract biological activity. Several studies have shown synergistic, additive or antagonistic effects that result from the presence of a multitude of compounds [285]. The extracts obtained in this work revealed a high content of carbohydrates, that could reach up to 30% of the total extract weight. The presence of low molecular weight compounds, such as sugars, with low biological potential, might therefore promote a decrease of the biological activity of the EtPUAE extract. To clarify this issue, the carbohydrates present in the extract need to be selectively removed, while cynaropicrin and other compounds with potentially high biological activity are retained.

Plant extracts fractionation and/or isolation of target compounds, namely cynaropicrin, is typically done using solvents with diverse selectivity and affinity to target compounds, using chromatographic processes and traditional operations such as distillation. However, distillation requires generally high temperatures, which is unappropriated for heat-sensitive products, and requires a high energy consumption (even if operated at reduced pressure) [36, 41, 43, 191]. In recent years, nanofiltration has been successfully applied for fractionation and concentration of different compounds [49-51, 56, 286] and, to the best of our knowledge, this is the first study regarding the removal of sugars and concentration of cardoon PUAE ethanolic extracts.

This work aims the selection of the best membrane and operating conditions for the separation of sugars (mainly glucose and fructose) from cynaropicrin, taking into account the molecular weight of these compounds (180 g mol^{-1} for glucose and fructose, and 346 g mol^{-1} for cynaropicrin). Since the extracts were produced with ethanol, it was also necessary to understand how the various membrane candidates behave and perform in the presence of the solvent. Attention will be given to the impact of ethanol on their performance, in terms of permeability and rejection behaviour.

Diananofiltration consists in the continuous addition of fresh solvent to the feed solution at the same rate as the permeate is recovered, aiming for a close-to-total depletion of less retained compounds, usually the ones with lower molecular weight [287]. References to diananofiltration using solvent resistant nanofiltration (SRNF) membranes, in a total organic solvent environment, is still scarce [50, 51, 56]. In this study, diananofiltration was therefore used for fractionation of cardoon PUAE extracts, in order to remove sugars (glucose and fructose) that are recovered in the permeate stream, assuring a maximum loss of 10% - 15% of cynaropicrin. The final retentate obtained, after concluding the diananofiltartion process, was then concentrated with the same membrane till a maximum volume reduction factor of 2. This overall procedure was defined with the objective of producing a cardoon extract rich in cynaropicrin with a low sugar content, for the nutraceuticals market, and a permeate stream rich in sugars for the food industry, following a concept of zero discharge. The biological potential of the fractionated extract was assessed and compared with the corresponding crude extract. An economic assessment of the overall integrate process was also conducted, to evaluate the potential for an industrial scale application.

5.3. Materials and Methods

5.3.1. Reagents and standards

The extraction solvent used was ethanol absolute ≥ 99.8% provided by BDh Prolabo (France). Acetonitrile (HPLC grade) and H_2SO_4 (95 – 97%) were both provided by Merck (France). Standard of cynaropicrin (≥ 95%) was acquired at Extrasynthese (France) and glucose (≥ 99.5%) and fructose (≥ 99%) were obtained from Sigma (Germany).

5.3.2. Raw material

Cynara cardunculus L. (DC) leaves were collected in June 2015 at the Experimental Center of Agriculture School of Instituto Politécnico de Beja, Portugal and preserved at -80 ºC. Before extraction, samples were air dried at room temperature till constant weight. Dried leaves were grounded using a domestic mixed grinder (Moulinex) in order to obtain a powder with particles presenting a size lower than 500 µm.

5.3.3. Extraction

Pulsed Ultrasonic Assisted Extraction (PUAE) experiments were performed using an UAE device (Bandelin HD3200, Germany), 20 kHz, equipped with a titanium alloy (Ti-AI6-V4) probe with 13mm diameter (Bandelin, Germany). Extraction was performed as described by Brás et al.[284]. Briefly, ethanolic PUAE, with a duty cycle of 25%, a solid/liquid (S/L) ratio of 1/27 (g/mL), extraction temperature of 44 ºC and amplitude of 67% (54.3 µm) was performed during 30 min. After extraction, the extract was filtered with a glass filter funnel, with porosity G4 and a 10-16 µm maximum nominal pore size. Extracts were stored at 4 ºC till processing by nanofiltration.

5.3.4. Nanofiltration experiments

5.3.4.1. Experimental setup

The nanofiltration experimental setup used for membrane selection is shown in Figure 5.1. It is comprised by a GE-Sepa CF cross-flow module (GE Osmonics, USA) and a high-pressure feed pump (Hydra-cell model G13, Wanner Engineering Inc., USA). The effective membrane area used was 140 cm^2.

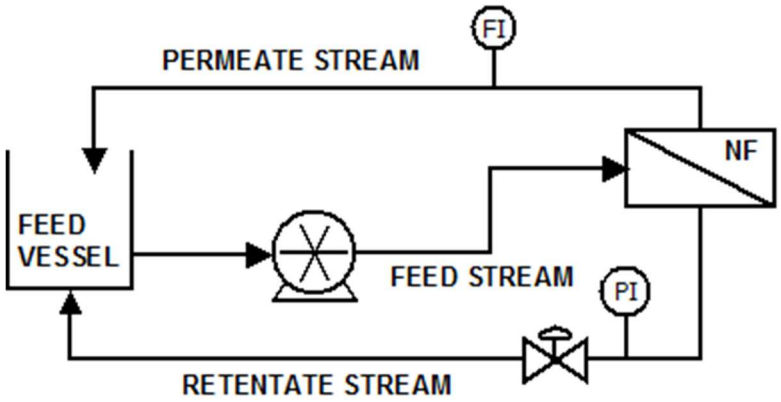


Figure 5.1. Experimental nanofiltration setup (NF) used for membrane selection. PI and FI are pressure sensors and flow-rate meters, respectively (adapted from Brás et al. [261]).

Considering the molecular weight of the target compounds, NF90 (Dow, USA) and NF270 (Dow, USA) membranes were pre-selected, taking into consideration their properties (see Table 5.1). Additionally, an organic solvent nanofiltration (OSN) membrane, DuraMem® 200 (Evonik, Germany) was also tested (Table 5.1).

Table 5.1. Characteristics of NF90, NF270 and DuraMem® 200 membranes

	NF90	NF270	DuraMem® 200
Manufacturer	Dow/Filmtec	Dow/Filmtec	Evonik
Surface material	Polyamide	Polyamide	Modified Polyimide
Molecular Weight cut-off (Da)	200 [288]	400 [289]	200 [290]
Maximum temperature (°C)	45	45	50
Maximum pressure (bar)	41	41	60
Water contact angle (°)	42.3±2.1 [291]	30.1±0.8 [291]	72.9 ± 2.7 [292]

5.3.4.2. Membrane selection

The membranes NF90, NF270 and DuraMem®200, were firstly characterized in terms of their permeability towards ethanol and the ethanolic extract. The membranes' behavior in terms of swelling in the presence of the solvent was also evaluated. Finally, the performance of these membranes was assessed concerning their rejection to the compounds under study (cynaropicrin, glucose and fructose). During permeability and rejection experiments, the system was operated under a total recirculation mode (Figure 5.1), for transmembrane pressures ranging between 4 and

20 bar, at 30 ºC, controlled by an external refrigeration bath (Model 89203-012, VWR International, USA). Samples of permeate and feed were collected for each pressure value and cynaropicrin, glucose and fructose concentrations were quantified by HPLC, in order to determine their rejection.

Permeability and rejections were calculated using the following equations, respectively:

$$J_v = L_p(\Delta P - \Delta\pi) \quad \text{(equation 5.1)}$$

$$R_i = \left(1 - \frac{C_{i,p}}{C_{i,f}}\right) \times 100 \quad \text{(equation 5.2)}$$

Where J_v is the solvent volumetric flux (L.m^{-2}.h^{-1}), Lp, is the membrane permeability (L.m^{-2}.h^{-1}.bar), ΔP is the transmembrane pressure (bar) and Δπ is the osmotic pressure difference (bar), which was determined by the Van't Hoff equation. R_i is the apparent rejection of solute i (%), and $C_{i,p}$(g.m^{-3}) and $C_{i,f}$ (g.m^{-3}) are the concentrations of solute i in the permeate and the feed, respectively.

Pre-weighed pieces (10 x 10 mm) of dry membrane were immersed in EtPUAE extract and allowed to stand at 30 ºC during 24h. After this procedure, they were removed, the liquid in excess was wiped and then the membranes' pieces were weighted again. The swelling degree of the membranes was, expressed in terms of weight of the membrane, was calculated by:

$$S_D = \frac{W_S - W_D}{W_D} \times 100 \quad \text{(equation 5.3)}$$

Where S_D is the membrane swelling degree (%), W_D (g) is the weight of the dried membrane and W_S (g) is the weight of the membrane after 24 h of contact with EtPUAE.

5.3.4.3. Diananofiltration

During diananofiltration experiments, the permeate stream was continuously removed and compensated by the addition of ethanol at the same flowrate (Figure 5.2). A feed volume of 1.5 L, a recirculation flow of 240L/h and a transmembrane pressure of 20 bar were used. The feed volume V_f (L) was kept constant by the continuous addition of ethanol, V_{EtOH} (L) and the number of Diavolumes, D (-), was calculated as the ratio between the fresh solvent volume added, V_{EtOH}, and the initial feed volume, Vf. Feed samples, and cumulative and instantaneous permeate samples were collected over time. Cynaropicrin, glucose and fructose concentrations were measured, and their apparent rejections calculated (equation 5.2).

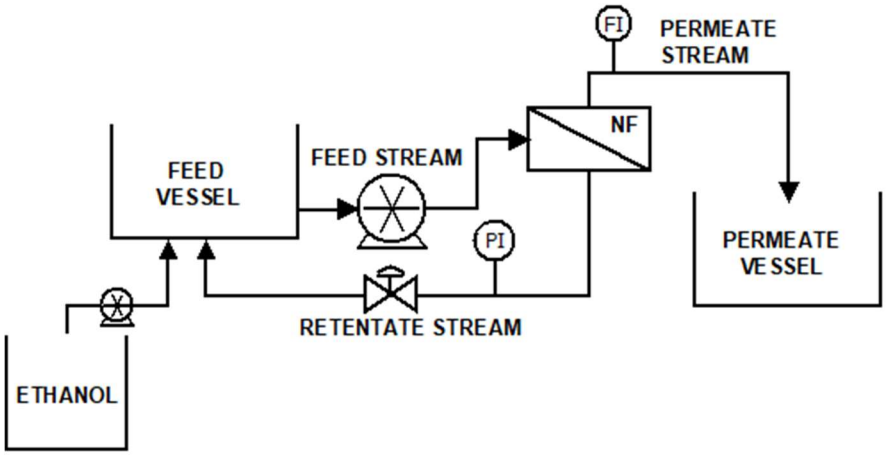


Figure 5.2. Experimental nanofiltration setup (NF) used for diananofiltration experiments. PI and FI are pressure sensors and flow-rate meters, respectively.

When operating in a diafiltration mode, the concentration of given compound i in the feed vessel, can be obtained by a mass balance to the system:

$$\frac{d(V_f C_{i,f})}{dt} = -J_v A C_{i,p} \qquad \text{(equation 5.4)}$$

Thus,

$$C_{if}\frac{dVt}{dt} + V_f\frac{dC_{i,f}}{dt} = -J_v A C_{i,p} \qquad \text{(equation 5.5)}$$

Since the volume is constant, Eq. 4 can be written as

$$\int_{t=0}^{t=t} dt = -\frac{V_f}{AJ_v}\int_{C_{i,f,t=0}}^{C_{if,t=t}} \frac{1}{C_{if}(1-R)} dC_{i,f} \qquad \text{(equation 5.6)}$$

In this case, to simplify, the apparent rejection (R) was considered to be constant during the experiment. So Eq. 5, can be solved as:

$$C_{i,f} = C_{i,f_o}\, exp\left(-\frac{J_v A(1-R)}{V_f} t\right) \qquad \text{(equation 5.7)}$$

Where, V_f is the feed volume (L), $C_{i,f}$ is the concentration of solute i in feed (mol.m^{-3}), $C_{i,p}$ is the concentration of solute i in the permeate (mol.m^{-3}), J_v is the permeate volumetric flux (L.m^{-2}.h^{-1}), A is the membrane area (m^2) and t (h) is the diafiltration time.

5.3.4.4. Nanofiltration

In order to increase the final concentration of cynaropicrin, a post-concentration step was performed, where the permeate was continuously removed until a pre-defined volume reduction factor, VRF (-), was achieved (Figure 5.3). In this work, a final VRF of 2 was achieved. Higher VRF values may be targeted but, since permeability was constant in this study, it was pointless to extend this operation. The VRF values during the concentration experiment were calculated using the following equation:

$$VRF = \frac{V_r}{V_f} \qquad \text{(equation 5.8)}$$

Where, V_f is the feed volume in the beginning of the experiment (m^3) and V_r is the volume in the feed circuit along time (retentate volume, m^3).

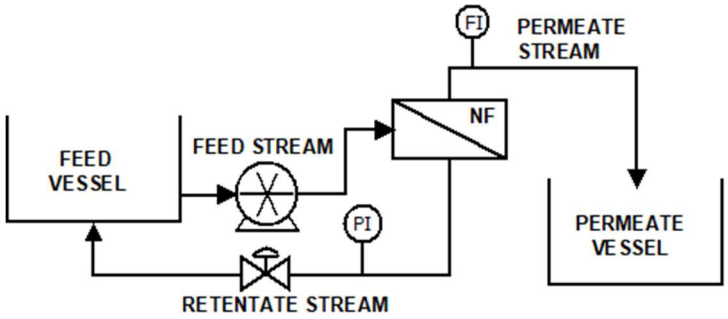


Figure 5.3. Experimental nanofiltration setup (NF) used for nanofiltration experiments. PI and FI are pressure sensors and flow-rate meters, respectively.

5.3.5. Cynaropicrin quantification by High Pressure Liquid Chromatography (HPLC)

Cynaropicrin was quantified by HPLC. A Dionex Ultimate 3000 system (Thermo Scientific, USA), equipped with a Diode Array detector DAD-3000 (Thermo Scientific, USA) was used. A Kinetex F5 2.6μ (4.6 x 150 mm) column, from Phenomenex (USA), was used at 30 ºC, with a water:acetonitrile volumetric ratio of 75:25 as mobile phase, at a flow rate of 0.5mL/min. All samples were pre-filtered with 0.22 μm pore size membrane filters (Pall, USA).

5.3.6. Monosaccharides quantification by High Pressure Liquid Chromatography (HPLC)

Glucose and fructose were also quantified by HPLC. A Dionex Ultimate 3000 system (Thermo Scientific, USA) was used, equipped with a refraction index ERC RefractoMax 520 detector (Thermo Scientific, USA). An Aminex HPX-87H (7.8 x 300 mm) cation exchange column, from Bio-Rad (7.8 x 300 mm) was used at 50 ºC, using a 5mM H_2SO_4 solution as mobile phase at a flow rate of 0.6 mL/min. All samples were pre-filtered with 0.22 μm pore size membrane filters from Pall, USA.

5.3.7. Total phenolic content quantification

Total phenolics were determined by the Folin-Ciocalteau assay. An aliquot of 10μL of sample extract was added to 150μL of a solution of 10% (v/v) Folin–Ciocalteu reagent. The mixture was stirred and allowed to stand in the dark for 5 min, before addition of 100μL ml of 60g/L Na_2CO_3. The mixture was then stirred and allowed to stand in the dark for 60 min, before reading at 725 nm on a microplate reader (MultiScan FC, Thermo Scientific, USA). A calibration curve was prepared using gallic acid and the results were expressed as mg of GAE (Gallic Acid Equivalent) per mL of extract.

5.3.8. Cell viability assay

Cell viability was determined with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay. Stock solutions of initial and fractionated EtPUAE extracts and pure cynaropicrin were prepared in ethanol. Human normal fibroblasts, BJ-5ta cell line (obtained from ATCC - American Type Culture Collection), were plated at a density of $1x10^5$ cells/well on a 96-well tissue culture plate, and incubated for 24h at 37 °C in a humidified atmosphere with 5% CO_2 (Sanyo

Electric CO, Japan). Then, cells were exposed to different concentrations of initial and fractionated EtPUAE (0.5 – 50 µg/mL), as well as to pure compound, cynaropicrin (0.5 – 10 µg/mL), dissolved in ethanol. Death and life controls were incubated with H_2O and culture medium (both 100% of the total volume), respectively. Solvent control corresponded to the percentage of ethanol present in the highest EtPUAE concentration tested.

After of 24 h, 48 h and 72 h of contact, the cell metabolic activity was assessed by the MTT viability assay. After incubation with samples, 10% MTT reagent (0.5 mg/ml) dissolved in medium was added to each well, and cells were incubated at 37 °C for 2 h. The MTT solution was carefully aspirated, and formazan crystals were dissolved with a DMSO/EtOH (1:1(v/v)) mixture. Absorbance was measured in a microplate reader (SpectraMax Plus, Molecular Devices, USA) at 570 nm. All the results were normalized relatively to the life control, which was considered as 100% of viability. The IC_{50}, defined as the sample concentration necessary to cause 50% inhibition of cell viability, was calculated by plotting the percentage of cell viability as a function of the sample concentration logarithm. Triplicates were performed in three independent experiments for each treatment and the data were expressed as mean ± standard deviation. The IC_{50} values (µg/mL) obtained for initial and fractionated extracts, at 24 h of incubation period, were analyzed using the Prism version 6.00 for Windows (GraphPad Software, USA). Where differences did exist, the source of the differences at a $p < 0.05$ of significance level was identified by all pairwise multiple comparison procedure. The Tukey's test was used for pairwise comparisons.

5.4. Results and discussion

5.4.1. Extracts characterization

Cynara cardunculus leaves were extracted by ethanolic PUAE and their chemical characterization was performed in terms of cynaropicrin, glucose, fructose and total phenolic content. The results obtained are presented in table 5.2.

Table 5.2. EtPUAE extract chemical composition

Compound	Concentration w/v (mg/mL)	Concentration w/w (mg/g dry extract)
Cynaropicrin	1.714	439.4
Glucose	0.121	31.1
Fructose	0.071	18.1
TPC	0.149*	38.2

*For Total Phenolic content (TPC), concentration is expressed in mgGAE/mL and mg GAE/ g dry extract for concentration (w/v) and w/w, respectively.

In a perspective to increase the biological potential of the EtPUAE extract, considering cynaropicrin potential and synergistic effects, the presence of compounds with a low biological potential, such as sugars, may promote a decrease of the overall activity. Therefore, the removal of sugars, with a

total quantification of 49.2 mg/g dry extract, is required to increase cynaropicrin purity in the final extract.

5.4.2. Membrane selection

Membrane characterization in terms of their permeability, swelling degree and solutes' rejection allows for understanding their behavior when in contact with EtPUAE extracts. Thus, aiming to produce an extract with a low content of sugars and enriched in cynaropicrin, the analysis of these parameters allows for the choice of the most suitable membrane.

The membranes under study, NF270 and NF90, are described as hydrophilic membranes with water contact angles of 27-30º and 53-57º [293]. Experimental results show that, when in contact with ethanol for 24 h at 30 ºC, they exhibit a swelling degree of 51.63% and 41.35% (w/w), respectively. Some studies assume that hydrophilic membranes, when in contact with ethanol, may suffer a reorganization of their structure due to clustering of hydrophilic and hydrophobic zones present in the active layer [294]. Both NF90 and NF270 membranes present a relatively high swelling in the presence of ethanol and the ethanol-based extract. Their stability in these media seems acceptable for operation and no integrity damage (such as pealing) was observed. Interestingly, when processing the ethanolic extract, their permeability lowers suggesting that other effects may be affecting their internal structure, in addition to the presence of ethanol. In what concerns their rejection behavior, in spite the extensive swelling observed, the tighter NF90 membrane presents the highest rejections but, the difference in rejection between cynaropicrin and the sugar compounds is too small. As a consequence, if this membrane is used, a high loss of cynaropicrin will be occurring if we keep the objective of removing most of the sugars (because a large number of diafiltration volumes will be required). On the other hand, the selection of the NF270 membrane is totally overruled because its rejection to cynaropicrin is too low.

The DuraMem® 200 membrane, commercialized as a solvent resistant membrane, presents an even lower permeability to ethanol and the ethanolic extract but, on the other hand, its low swelling in ethanol suggests a high stability in this solvent. Even more relevant is the fact that it presents a very high rejection to cynaropicrin and a sufficiently lower rejection for glucose in fructose. Considering this rejection behavior, it may be anticipated a high retention of cynaropicrin (with a very low loss to the permeate), while the sugar compounds will be washed to the permeate, if sufficient diafiltration volumes are use.

Previous studies with NF270 and NF90 for glucose rejection in aqueous solutions, have shown high glucose rejection values (96% for NF270 and 98% for NF90) and low swelling degree [295] in comparison to the ones obtained for the ethanolic extract on the present study (26% and 86% for NF270 and NF90 membranes, respectively). These differences may be attributed to a higher swelling, observed in ethanolic media.

On what concerns the DuraMem® 200 membrane, characterized as an amphiphilic membrane (see water contact angle of 72.9º), its permeability to ethanol is rather low (0.76 $L/m^2.h.bar$) comparatively to the others membranes but its swelling degree of 17.12% was the lowest one, with

high solutes' rejection, corresponding to what was expected due to the fact that it is an organic solvent resistant membrane and its low MWCO (200 Da).

Table 5.3. Values for ethanol and extract permeability, EtPUAE cynaropicrin, glucose and fructose, rejections at 20 bar and $swelling_{extract}$ for the NF90, NF270 and DuraMem® 200 membranes.

Membrane	Lp_{EtOH} ($L/m^2.h.bar$)	$Lp_{Extract}$ ($L/m^2.h.bar$)	R_i (%) (20 bar)			$Swelling_{extract}$ (w/w)
			Cynaropicrin (MW 346.38 $g.mol^{-1}$)	Glucose (MW 180 $g.mol^{-1}$)	Fructose (MW 180 $g.mol^{-1}$)	
NF90	3.15	0.65	93	86	83	41.35 ± 2.86%
NF270	2.30	0.76	32	26	24	51.63 ± 2.68%
DuraMem® 200	0.76	0.25	98	81	76	17.12 ± 1.53%

Membrane selection for subsequent experimental diananofiltration studies took into consideration the comments above, supported by a quantitative analysis using the mass balance described by equation 7, which allows for prediction of membrane behavior during diananofiltration. The mass balance was applied considering a feed volume V_f of 1.5L and the objective of a maximum loss of 10% for cynaropicrin. The predicted results are shown in Table 5.4. The optimal separation is the one that promotes the highest removal of "contaminants" (glucose and fructose) and the lowest loss of the target compound (cynaropicrin). Considering a maximum allowed depletion of 10% for cynaropicrin, prediction calculations for a diananofiltration experiment show that a removal of 58% and 62% of glucose and fructose, respectively, could be achieved with a DuraMem® 200 membrane, contrarily to the removal of 22% of glucose and fructose using a NF90 membrane.

Table 5.4. Predicted results for the compounds under study (cynaropicrin, glucose and fructose) for the NF90 and the DuraMem® 200 membranes, where $W_{loss,i}$ (%) corresponds to the percentage of the compound i weight loss and V_D corresponds to the predicted diavolumes for a maximum 10% cynaropicrin weight loss.

Membrane	$W_{loss,\ cyn}$ (%)	$W_{loss\ glucose}$ (%)	$W_{lossl,\ fructose}$ (%)	V_D
NF90	10	22	22	1.82
DuraMem® 200	10	58	62	5

From these calculations, we anticipate that the DuraMem® 200 membrane will lead to a much better cynaropicrin/sugars separation and, therefore, it was selected for the diananofiltration experiments with the EtPUAE extract.

5.4.3. Diananofiltration experiment

5.4.3.1. Permeate flux during diananofiltration

The *Cynara cardunculus* ethanolic leaves extract was processed in a diananofiltration mode at a transmembrane pressure of 20 bar and 35 ºC. Figure 5.4 a) and b), shows the evolvement of the permeate flux with time and with the diavolumes of fresh solvent added, respectively. It is possible to observe that after an initial flux increase and, after 24h of operation, it reaches a plateau. A similar behavior was observed by Teixeira et. al. when using organic solvent nanofiltration membranes for

the purification of steryl esthers in hexane. That work reports a permeate flux increase along with the addition of fresh solvent (and time), which was related to a progressive membrane swelling and to a decrease of the solution viscosity, as several low molecular weight compounds (e.g. sugars) are washed and recovered in the permeate [50].

In order to understand the influence of the permeate flux increase, the apparent rejections of the compounds under were determined, but no significant evolvement was observed (Figure 5), showing that the increase of permeate flux did not negatively influenced the rejection of solutes. Therefore, the previous assumption of constant apparent rejection for the compounds under study, referred previously, can be considered adequate. For a correct diananofiltration simulation, the permeate flux evolvement, J_v, was considering the data presented in Figure 4; the mathematical fit to the experimental data gives:

$$J_V = 7.333 - 3.048e^{-0.0755t} \quad \text{(equation 5.9)}$$

with an experimental fit error of 0.9053.

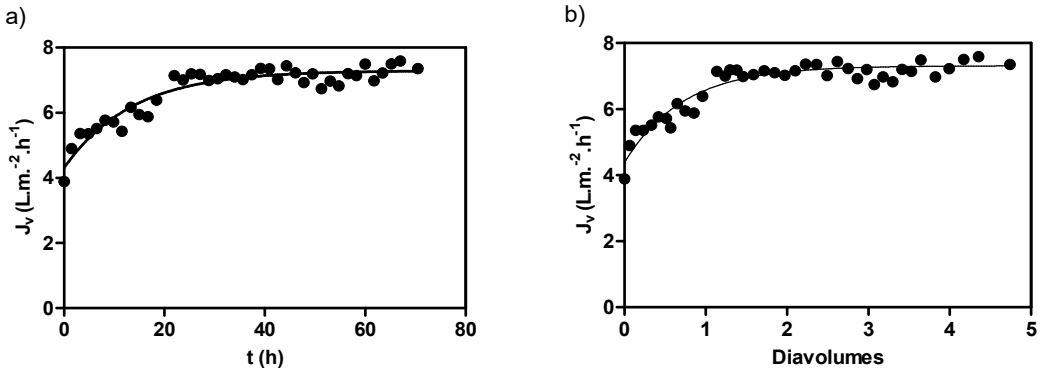


Figure 5.4. Experimental variation of permeate flux, J_v (L.m^{-2}.h^{-1}) as a function of a) time and b) number of diavolumes, for the DuraMem® 200 membrane, during a diananofiltration experiment, where ●corresponds to experimental values and line corresponds to the model fit.

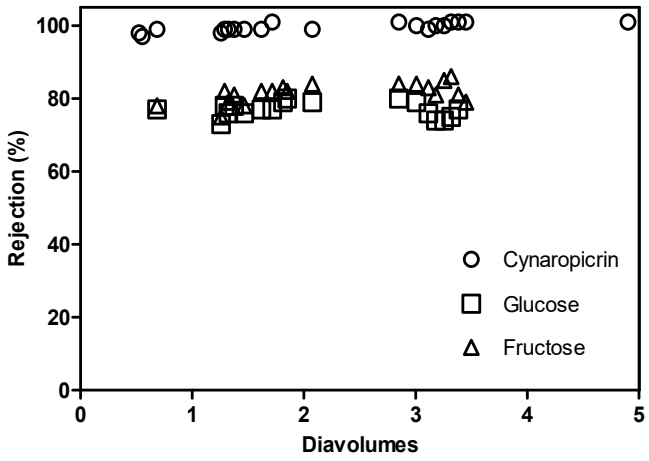


Figure 5.5. Experimental apparent rejection (%) for cynaropicrin, glucose and fructose plotted against the number of diavolumes, during the diananofiltration operation.

5.4.3.2. Solute concentration during diananofiltration

Diananofiltration experiments were performed aiming a maximum removal of sugars with a minimum loss of cynaropicrin.

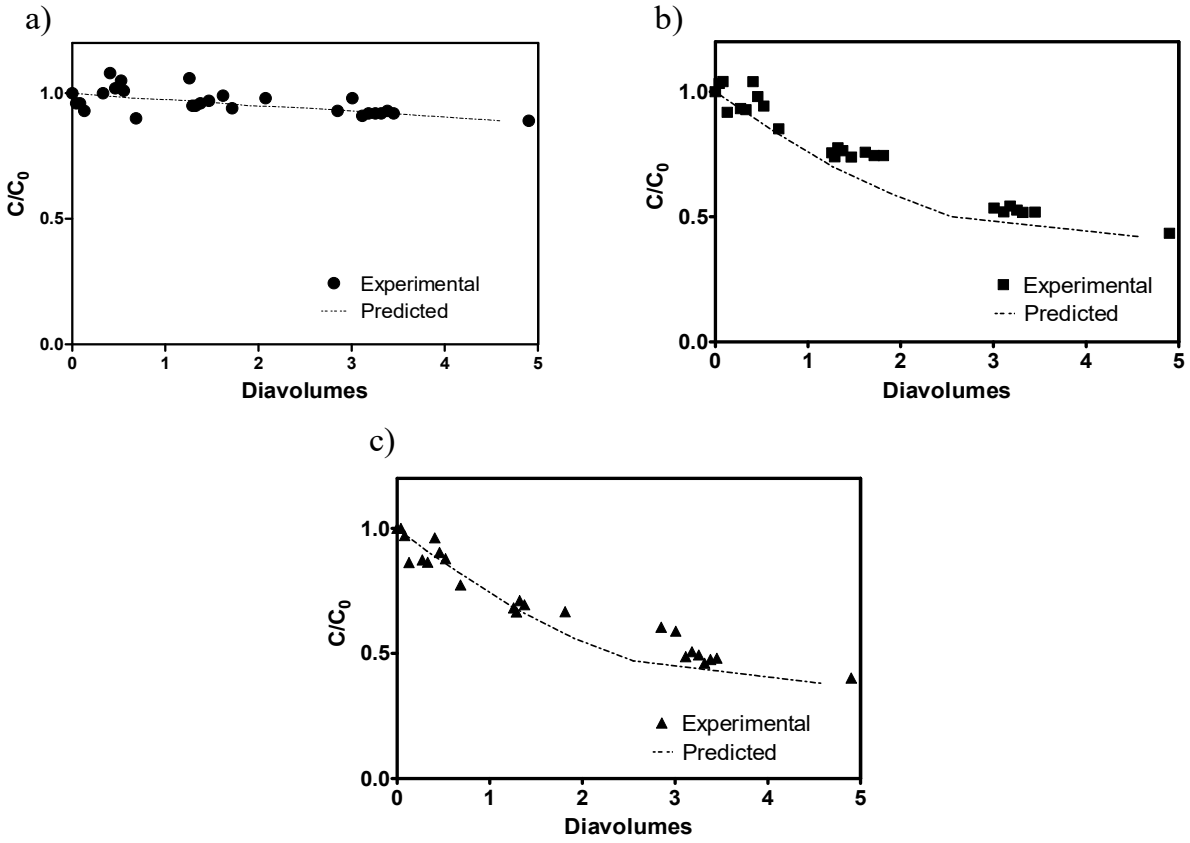


Figure 5.6. Experimental and predicted normalized solute concentration in the retentate, as a function of the number of diavolumes. a) cynaropicrin; b) glucose; c) fructose.

Comparison between experimental data and the predicted ones, according to equation 5.7, and assuming constant rejection for each solute, shows that the predicted ones describe well the experimental data obtained (Figure 5.6). Also, a removal of 56.73% and 64.03% of glucose and fructose was achieved with a maximum cynaropicrin weight loss of 9.30% at 4.74 diavolumes (Table 5.5).

Table 5.5. Comparison between cynaropicrin, glucose and fructose retention / removal (experimental values and predicted by equation 7) for the diananofiltration experiments

Compound	Initial mass (mg)	Final mass in the retentate (mg)		Experimental mass loss (%)
		Experimental	Predicted	
Cyn	2194	1990	1958	9.3%
Glucose	342	148	141	56.7%
Fructose	183	64	70	64.0%

5.4.4. Nanofiltration

In order to increase the cynaropicrin concentration on the final purified extract, a concentration step, by nanofiltration, was applied up to a volume reduction factor, VRF, of 2. Figures 5.7 and 5.8 represent the variation of the permeate flux and cynaropicrin normalized concentration, respectively.

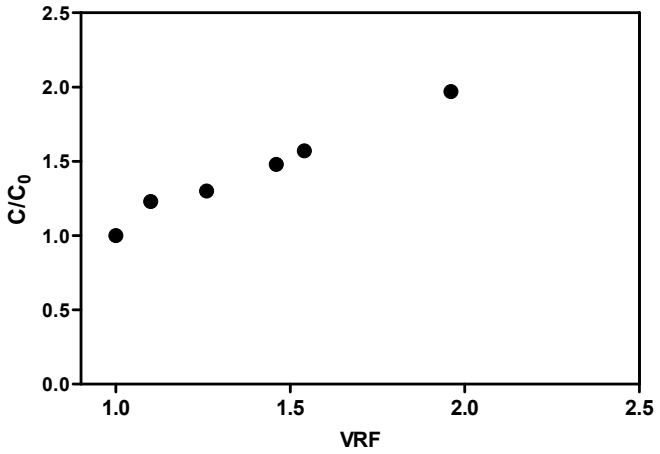


Figure 5.7. Normalized cynaropicrin concentration as a function of the volume reduction factor (VRF) obtained by nanofiltration (ΔP = 20 bar), after extract washing by diananofiltration

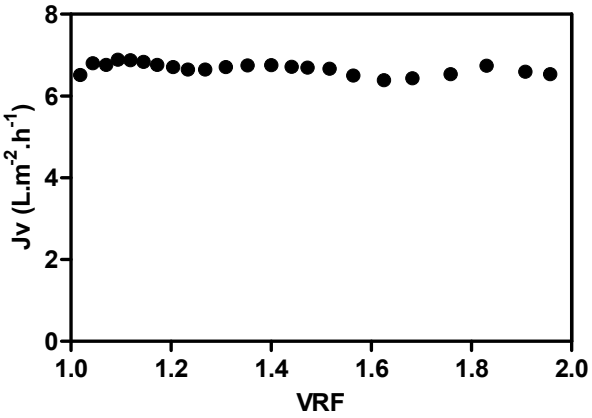


Figure 5.8. Permeate flux as a function of the volume reduction factor (VRF) obtained by nanofiltration (ΔP = 20 bar), after extract washing by diananofiltration.

During the concentration process by nanofiltration, no decrease of the permeate flux was observed (figure 5.8), which are excellent news. A mass balance to the entire system shows that at the end of the nanofiltration experiment, there was a decrease of approximately 57% and 87.5% on glucose and fructose content, achieving a final removal of glucose and fructose of 93.0% and 95.6%, respectively (Table 5.6). The final loss of cynaropicrin reaches a value of 13.9%, which is an excellent result.

Table 5.6. Global experimental cynaropicrin, glucose and fructose mass loss

Compound	Mass (mg)		
	Initial	Final	% mass loss
Cyn	2194	1890	13.9%
Glucose	342	24	93.0%
Fructose	183	8	95.6%

5.4.5. Cell viability assay

With the removal of low molecular weight compounds, as sugars, it was expected an increment of the extract biological activity [51, 286]. The biological activity was assessed with human normal skin fibroblasts, BJ5-ta cell line, through MTT assays performed with the initial and fractionated extracts, as well as with cynaropicrin, the major solute in initial and fractionated EtPUAE extracts, with a content of 439 and 517 mg/g dry extract, respectively.

Based on the results obtained for cynaropicrin IC_{50} (10.32 ± 1.3 $\mu g_{cynaropicrin}$/mL) (Table 7), revealing an extremely active solute, we aimed to verify the impact of removing low molecular weight compounds, present in EtPUAE, on the extracts bioactivity. It is expected that the removal of these compounds increases cynaropicrin bioavailability and, consequently, potentiates the fractionated extract bioactivity.

IC_{50} values were predicted by calculation, correlating the cynaropicrin content within each extract, with the cynaropicrin IC_{50} value. According to the results presented in Table 5.7, the predicted IC_{50} values are identical, when comparing initial *versus* fractionated extracts. Based on this, theoretically, the removal of low molecular weight compounds, as sugars, would not influence the final bioactivity of the fractionated extract. However, the experimental IC_{50} for the initial extract (29.9 ± 4.2 $\mu g_{extract}$/mL) revealed to be higher comparatively to the fractionated extract (21.4 ± 2.2 $\mu g_{extract}$/mL) ($p < 0.05$) (Table 5.7), indicating an increase of biological activity, after removal of lower molecular weight compounds by diananofiltration. Moreover, when comparing the predicted and the experimental IC_{50} values, for the initial extracts, the expected bioactivity of the initial extract should be considerably higher (23.5 ± 1.2 $\mu g_{extract}$/mL) , which is not confirmed by the experimental IC_{50} value (29.9 ± 4.2 $\mu g_{extract}$/mL). This result indicates that the removal of compounds by diananofiltration impacts positively on the bioavailability of cynaropicrin, and consequently on the higher biological activity observed with the fractionated extract.

Table 5.7. IC_{50} values regarding the 24h cellular viability of initial and fractionated EtPUAE, as well as of cynaropicrin solution (in ethanol), on BJ5-ta cell line, determined through MTT assay.

Sample	IC_{50} ($\mu g_{cynaropicrin}$/mL)	Predicted IC_{50} ($\mu g_{extract}$/mL)	Experimental IC_{50} ($\mu g_{extract}$/mL)
Initial extract	-	23.5 ± 1.2^{b}	29.9 ± 4.2^{a}
Fractionated extract	-	19.9 ± 1.2^{b}	21.4 ± 2.2^{b}
Cynaropicrin	10.3 ± 1.3		-

Average ± standard deviation estimated from three extracts analyzed in triplicate.
Values with different superscripts, within the same column, are statistically different ($p < 0.05$)

5.4.6. Integrated process of extraction and fractionation

To the best of our knowledge, there are no studies aiming the separation of sesquiterpene lactones from monosaccharides, dissolved in an organic solvent, such as ethanol. Considering a future integration of the overall process for cynaropicrin recovery from *Cynara cardunculus* leaves by PUAE, with the removal of sugars and low molecular weight compounds, an integrated process is here proposed. This process comprises also the recovery of ethanol from the permeate produced during diananofiltration, by evaporation. The ethanol recovered can be then reused as wash solvent during further diananofiltration operations.

Aiming to produce a final extract rich in cynaropicrin for the nutraceutical industry, a spray dryer unit was considered, due to the mild temperature conditions that may be used during operation (Figure 5.9).

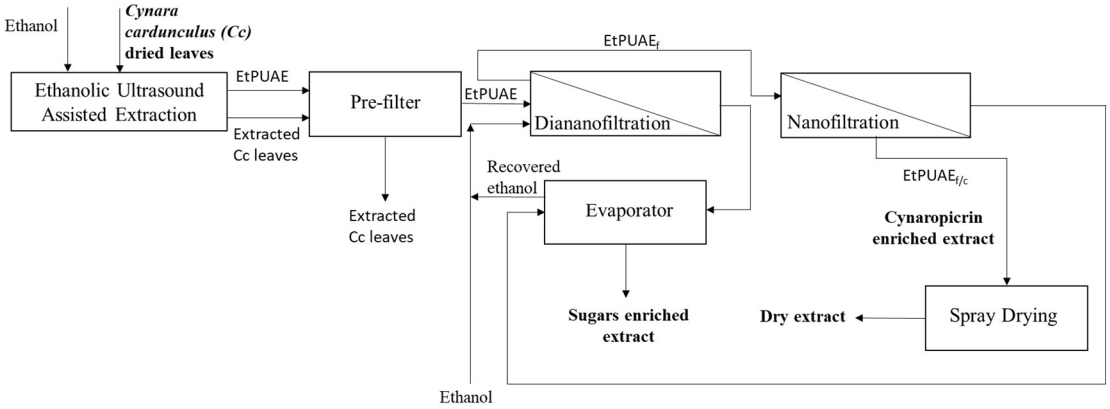


Figure 5.9. Integrated process for processing of *Cynara cardunculus* dried leaves

5.4.7. Economic assessment

An economic study was performed for a 10 ton/year supply of dried cardoon leaves, from a local farm, corresponding to a production of 0.8 ton/ha. According to our previous studies, the extraction process is accomplished by PUAE, followed by a filtration with a filter press, before fractionation by membrane processing [122]. Considering the results obtained in sections 3.3 and 3.4 the nanofiltration equipment should be installed with DuraMem® 200 membranes and operated at 20 bar of transmembrane pressure [296]. According to results obtained, it was considered a cynaropicrin concentration at the end of the diananofiltration and nanofiltration experiment of 517 mg / g dry extract and a dry extract production of 52 mg extract/ g Dry weight $_{biomass}$. From that, an input of 10 ton/year of dried cardoon leaves with a production of 52 $kg_{extract}$ / ton dried cardoon leaves with a cynaropicrin concentration of 517mg/g extract was considered for the economic assessment.

An operating regime of 48 weeks/year, 7 days/week and 24h/day was considered. Integrated process was operated in sequential batches with a single batch involving a sequence of unit operations (Figure 5.10). The average number of batches per day was determined in order to maximize the use of the equipment with the highest occupation time: the nanofiltration unit (5 h of

operation followed by 2 h of cleaning (Table 5.8), allowing an average number of 3.4 batches per day.

Table 5.8. Equipment operation and cleaning time during one batch for the cynaropicrin integrated process.

Equipment	Operation t (h)	Cleaning t (h)
UAE Extractor	1	1
Pre-Filter	1	1
Membrane unit (diananofiltration)	4	2
Membrane unit (nanofiltration)	1	
Spray Dryer	5	1

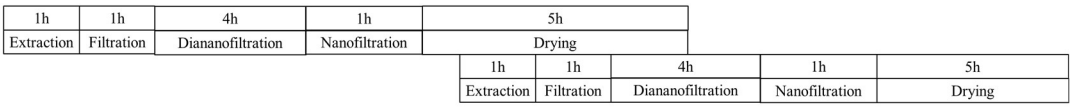


Figure 5.10. Sequence of each stage operation during 24h, for the cynaropicrin integrated process

The economic study for the production of cynaropicrin enriched extract is presented in table 5.9. The recovery of the ethanol used was considered, at a reuse rate of 80% per batch. The overall process comprises the following equipments: ultrasound extractor, filter press, nanofiltration unit, solvent evaporator and spray drying, as well as eight storage tanks. Due to the high cost of the spray dryer unit, during the final concentration step, a concentration factor of 10 was considered, instead of the concentration step of 2, obtained at laboratory scale. The cost of the ultrasound extractor and the nanofiltration unit was obtained from specialized equipment suppliers. Costs of the remaining equipment were estimated based on equipment costs given by Matche [297].

From the economical evaluation obtained and presented in table 5.9, the investment cost represent 60% of total costs, with main equipments being 28% of the total investment. According to the information gathered, the ultrasound extractor and the solvent recovery evaporator equipment represent 27% and 33% of the equipment investment, respectively.

Regarding direct costs, manpower costs consisted on four shifts with two workers per shift at 21 000 €/person/year. It was also considered al time (8 h/day) production supervisor and a maintenance technician per shift at 35 000 €/person/year each. Regarding electric consumption, a 0.1 €/kWh price was considered, with energy costs representing 2% of direct costs. A membrane lifetime of 3 years was considered, which represents an annual cost of 800 €. Based on the selling price provided by a local supplier, the biomass cost, dried and milled *Cynara cardunculus* leaves, was considered to be 4 €/kg. Although the natural plant extracts market for nutraceutical and food industries is well established, information regarding selling price for *Cynara cardunculus* extracts and cynaropicrin extracts is scarce. A value of 2500 €/$kg_{extract}$ was assumed, considering the correlation between cynaropicrin concentration (518 mg/$g_{extract}$) and the selling price in pure form [298]. From this study, a calculated pay-back period of 4.58 years was estimated.

Table 5.9. Economic evaluation of the integrated process for the production of cynaropicrin rich extract.

Cynara cardunculus leaves processing capacity (TON/year)		10
Dry extract (kg/year)		520
Installed ultrasound assisted extractor (L)		**240**
Installed nanofiltration membrane		**(DuraMem® 200)**
Membrane Area (m^2)		**48**
$Flux_{diananofiltration}$ = $Flux_{nanofiltration}$ ($L/m^2.h$) at ΔP=20bar		**7**
Investments costs		
Total fixed investment cost (€)		**1 349 403.00 €**
	Total equipment costs (€)	377 800.00 €
	Assembly (35% total equipment cost) (€)	132 230.00 €
Capital costs (€/year)[a]		**183 341.00 €**
Total operational costs (€/year)		**577 453.00 €**
Fixed costs (€/year)[b]		29 687.00 €
Direct costs (€/year)		**477 281.00€**
	Cost of dried cardoon leaves (€/year)	40 000,00 €
	Manpower (€/year)	308 000.00 €
	Energy costs (€/year)	10 874.00 €
	Quality control (€/year)	30 800.00 €
	Maintenance (€/year)	53 976.00 €
Indirect costs (40% manpower and maintenance) (€/year)[c]		144 790.00 €
General expenses (10% of indirect costs) (€/year)		72 418.00 €
Total costs (capital + total operational) (€/year)		**907 516.00 €**
Revenues (€/year)		
Dry extract sales (2500€/kg) (€/year)		1 300 000.00 €
Annual return		
	Return without taxes	392 484.00 €
	Taxes (25%)	98 121.00 €
Return after taxes (€/year)		**294 363.00 €**
Pay-back (year)		**4.58**

[a] Based on a depreciation over 10 years and 6% annual taxes [299]
[b] Includes amortization, local taxes and insurances
[c] Includes administration, sales and marketing costs [299]

In order to understand which parameter presents a higher impact on the pay-back period, a sensitivity analysis was performed, considering a variation of the biomass cost and of the selling price of dry extract (Figure 5.11). From the sensitive analysis, it was possible to observe that the dry extract selling price represents the key parameter, to which the pay-back period is more sensitive. The impact of the biomass cost is not relevant.

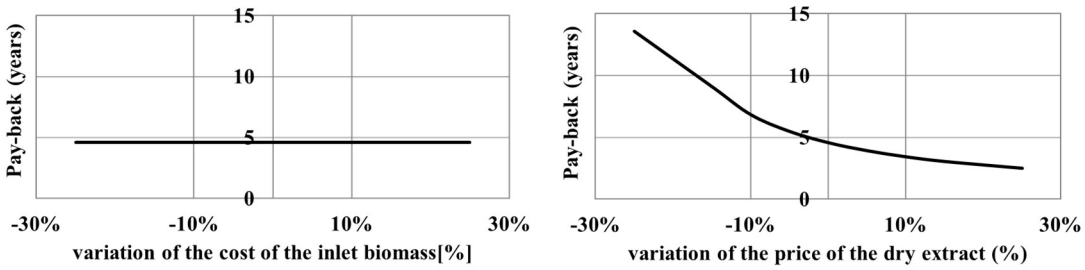


Figure 5.11. Impact of biomass cost and of extract selling price on the pay-back period of a plant for production of cynaropicrin rich extracts.

5.5. Conclusions

This work proposes an integrated process for the removal of carbohydrates from ethanolic extracts, obtained by pulsed ultrasound assisted extraction of cardoon leaves, by diananofiltration using a DuraMem® 200 membrane. The cynaropicrin loss in this operation is lower than 10% in mass after washing with 5 diavolumes. The extract obtained is then concentrated by solvent resistant nanofiltration, assuring an overall cynaropicrin mass loss of only 13.9%.

Cell viability analysis on human skin fibroblasts indicates an increase in the biological activity of the fractionated extract, that induces a cell viability reduction, mainly attributed to the removal of low molecular weight compounds, such as sugars.

An integrated process for the production of cynaropicrin enriched extracts is proposed. The economic analysis performed estimates a pay-k time of 4.58 years, for an annual production of cynaropicrin enriched extract of 520 kg/year.

6

Development of bioactive films based on chitosan and *Cynara Cardunculus* leaves extracts for wound dressings

Published as: Brás, T., Rosa, D., Gonçalves, A. C., Gomes, A. C., Alves, V. D., Crespo, J. G., Duarte, M.F., Neves, L. A. (2020). Development of bioactive films based on chitosan and *Cynara cardunculus* leaves extracts for wound dressings. International Journal of Biological Macromolecules. https://doi.org/10.1016/j.ijbiomac.2020.09.109

6.1. Abstract

The development of natural based, effective and protective wound dressings associated to local treatment applied on chronic wounds, represents a major challenge nowadays. In this work chitosan-based films were prepared with different concentrations of Ethanolic Pulsed Ultrasound Assisted Extracts from *Cynara cardunculus* leaves (EtPUAE). The physico-chemical film properties revealed that extract incorporation influences the volumetric swelling capacity and mechanical properties of the films, leading to materials with a lower fluid absorption capacity and more fragile. However, no impact was detected on the thermal properties of the films, as well as on their dense structure characterized by Scanning Electronic Microscopy (SEM) analysis. Biological screening of chitosan-based films show that chitosan with a 1% (w/w) and a 5% (w/w) EtPUAE loading did not induce cytotoxicity on normal human skin fibroblasts (Bj5-ta cell line), mainly attributed to cynaropicrin (extract key active compound) present in the film below its IC50 value. Nevertheless, chitosan-based films with 5% (w/w) EtPUAE presented an interesting anti-inflammatory activity. Bj5-ta cells stimulated with liposaccharides (LPS), presented a reduction of 86% on IL-6 cytokine levels, after exposure to chitosan with 5% EtPUAE film extract.

The results obtained in this study open up the possibility of successfully using chitosan films doped with EtPUAE for development of chronic wound dressings, with the advantage of using naturally-sourced materials with anti-inflammatory activity.

Keywords: Chitosan films; *Cynara cardunculus* extracts; cynaropicrin; wound dressing; skin inflammation

6.2. Introduction

Defined as a disruption resultant from physical or thermal damage, in the continuity of the epithelial lining of the skin or mucosa, a wound can be defined as acute or chronic according to its healing time [62]. Comprising a dynamic and complex process of tissue regeneration and growth, wound healing progresses through four different phases: coagulation and haemostasis, inflammation, proliferation and maturation [300]. Chronic wounds fail to progress through the normal stages of the healing cascade and are not repaired in an orderly and timely manner. Such failure may result from repeated tissue insults or underlying physiological conditions such as diabetes and malignancies, persistent infections, poor primary treatment, and other patient related factors, presenting a biological, psychological, social, and financial burden on both individual patients and, healthcare systems worldwide [301].

The development of advanced biomaterials for transdermal drug delivery is a growing area of biomedicine wherein intensive research is carried out, especially for chronic wound healing and/or efficient delivery of active pharmaceutical ingredients across skin. In fact, wound dressings are an important segment of the medical and pharmaceutical wound care market, with the Wound Dressing Market valued at USD 6309.62 billion in 2018 and expected to reach up to USD 11168.93 billion by 2025 with a Compound Annual Growth Rate (CAGR) of 8.50% over the forecast period, according to the Global Advanced Wound Dressing Market Report, by Brandessence Market Research and Consulting Pvt. ltd [83].

Considering a proper and timely wound healing, dressing selection should be mostly based on its ability to: i) provide or maintain moist environment; ii) enhance epidermal migration and promote angiogenesis, as well as connective tissue synthesis; iii) allow gas exchange between wounded tissue and the environment, towards maintenance of appropriate tissue temperature, in order to improve blood flow to the wound bed; iv) protect against bacterial infection; v) be non-adherent to the wound and easy to remove after healing; vi) provide debridement action to enhance leucocytes migration and support the accumulation of enzymes; and vii) be sterile, non-toxic and non-allergic [302].

Chitosan is one of the most studied biopolymers for wound dressings' application, mainly due to its biocompatibility and biodegradability, mostly arising from the presence of primary amines along the chitosan backbone [303]. Produced by chitin deacetylation, chitosan is a linear polysaccharide, composed of glucosamine and N-acetyl glucosamine units linked by b (1–4) glycosidic bonds. Chitosan is an excellent candidate for wound dressing applications due to its non-toxicity, antimicrobial and anti-inflammatory properties, as well as its capacity to stimulate haemostasis and accelerate tissue regeneration [69, 304, 305]. Chitosan can be processed into different forms, such

as hydrogels, scaffolds, sponges or films, and the latter are characterized by being thin, less obtrusive with the ability to act as a vehicle for transdermal drug delivery [306, 307].

Given their biological properties, safety and relatively low cost, bioactive plant extracts have been the subject of particular interest in the context of wound healing and transdermal drug delivery, namely as potential therapeutics for chronic wounds [58, 308-310]. Comprising more than 5000 compounds, sesquiterpene lactones (SL) are one of the major groups of plant secondary metabolites presenting a high biological potential, namely anti-inflammatory [35, 311-314]. Cynaropicrin, a guaianolide SL, has raised high scientific interest, due to biological activities such as regulation of major adhesion molecules CD29 and CD98 [25] and anti-inflammatory response [35]. Recently, Brás et al. described cynaropicrin extraction from *Cynara cardunculus* leaves and their optimization process using ethanol as solvent and Pulsed Ultrasound Assisted methodology (EtPUAE), followed by fractionation by membrane separation processes. [122, 284, 315].

To the best of our knowledge, this is the first report on the development and characterization of chitosan films doped with *Cynara cardunculus* leaves extracts, and evaluation of their potential use as wound dressing. In the present work, EtPUAE extracts from *Cynara cardunculus* leaves were incorporated into chitosan films, at different cynaropicrin/chitosan loadings (1, 5 and 10% (w/w)). The obtained films were characterized for their chemical and mechanical properties, as well as biological potential, with special focus on anti-cytotoxic and inflammatory activities.

6.3. Materials and Methods

6.3.1. Reagents and standards

Chitosan ≥ 75% deacetylation degree was provided by Sigma Aldrich (USA). Acetic acid > 99.8%, sodium chloride (> 99.5%) and potassium chloride (p.a.) were provided by Honeywell (USA), ethanol absolute ≥ 99.8% and potassium acetate were provided by BDh Prolabo (France), acetonitrile (HPLC grade) by Merck (France), and cynaropicrin was provided by Extrasynthese (France). Sodium bromide (99.0%) was purchased from Alfa Aesar (USA) Dimethyl sulfoxide (DMSO) cell culture grade was obtained from Applichem (Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide was purchased from Calbiochem (USA). Acridine orange was purchased from Invitrogen (USA). DMEM, Medium 199, fetal bovine serum (FBS) suitable for cell culture, bovine serum albumin (BSA) (> 96% purity), Lipopolysaccharides from Escherichia coli O111:B4 (LPS) and Piroxicam (≥ 98%) were purchased from Sigma Chemicals Co (Spain). Human IL-6 ELISA development kit (HRP) was purchased from Mabtech (Sweden).

6.3.2. Raw material

Cynara cardunculus L. (DC) leaves were collected in June 2016 at the Experimental Center of Agriculture School of Instituto Politécnico de Beja, Portugal and preserved at -80 ºC. Before extraction, samples were freeze-dried in a freeze-drier (Scanvac coolsafe, Labogene, Denmark).

Freeze-dried leaves, with moisture content below the detection limit of 1%, were grounded using a domestic mixed grinder (Moulinex, France), in order to obtain a powder constituted by particles with a size lower than 500 µm. After gridding, the freeze-dried powder was stored under vacuum at room temperature.

6.3.3. Cynaropicrin Extraction

Pulsed Ultrasonic Assisted Extraction (PUAE) experiments were performed using a 20 kHz UAE device (Bandelin HD3200, Germany), equipped with a 13mm diameter titanium alloy (Ti-AI6-V4, Bandelin, Germany). Extraction was performed as described by Brás et al. [284]. Briefly, pulsed UAE, with a duty cycle of 25%, a solid/liquid (S/L) ratio of 1/27 (g/mL), extraction temperature of 44 ºC and amplitude of 67% (54.3 µm) was performed during 30 min. After extraction, the ethanolic extract (EtPUAE extract) was filtered using a glass filter funnel, with porosity G4 and a 10-16 µm maximum nominal pore size. The solvent was evaporated at low pressure on a rotary evaporator (Hei-VAP Advantage, Heidolph, Germany) and dried extract was storage at room temperature till usage.

EtPUAE cynaropicrin content was determined by HPLC. A Dionex Ultimate 3000 system (Thermo Scientific, USA) equipped with a Diode Array detector was used. A Kinetex F5 2.6 µm (4.6 x 150 mm) column (Phenomenex, USA) was employed at 30 ºC, using as mobile phase a mixture of water and acetonitrile (75:25 (v/v)) with a flow rate of 0.5 mL/min. All samples were pre-filtered with 0.22 µm pore size membrane filters (Pall, USA).

6.3.4. Films Development

Chitosan films, neat or with incorporated EtPUAE extracts, were prepared by the solvent evaporation method and adapted from Ferreira et al. [316]. A chitosan solution was prepared by adding 2% (w/v) in acetic acid aqueous solution 1% (v/v) with magnetic stirring at 200 rpm until the chitosan powder was completely dissolved. The polymer solution was vacuum filtrated twice using Miracloth® (Merck Millipore) as filter.

Chitosan films doped with EtPUAE extracts were also prepared. For that, EtPUAE extracts on the proportion of 1, 5 and 10% (w/w) (cynaropicrin/chitosan) were firstly dissolved in 10mL of ethanol and then added to a 25mL filtered Chitosan solution. Chitosan and Chitosan with EtPUAE forming solutions were centrifuged at 5000 rpm for 15min to remove possible air bubbles. The solutions were then casted on polypropylene petri dishes (90 mm diameter) and dried at 40 ºC for 48h. Dried chitosan-based films were peeled off, weighted and equilibrated at 25 ºC with different relative humidity ambiences, to further understand the influence of water on the final properties of the films. Defined saturated salt solutions were prepared in four different desiccators by mixing inorganic salts with distilled water, in order to obtain a specific relative humidity (RH) as described in Table 6.1.

Table 6.1. Relative humidity of air in contact with specific saturated salts at 25 ºC [317].

Salt	Relative humidity (RH(%))
Potassium acetate	22.6
Sodium bromide	57.7
Sodium chloride	75.3
Potassium chloride	84.3

6.3.5. Characterization of Chitosan-based films

6.3.5.1. Scanning Electron Microscopy (SEM)

Surface and cross section images were used to detect morphology differences on chitosan films, with and without EtPUAE extracts. The films surface and cross section images were obtained with different magnifications, respectively 1000x and 3000x, using an analytical Scanning Electron Microscope Hitachi 5-2400 equipment (Hitachi Ltd, Japan), equipped with a field-emission electron source of 15 kV. Prior to observation, films were cut with liquid nitrogen, mounted on a metal scrub and coated with a thin layer film of gold-paladium.

6.3.5.2. Fourier Transform Infrared Spectroscopy (FT-IR) analysis

For the analysis of possible interactions established between EtPUAE extract components and chitosan, FT-IR spectra analysis was performed. FT-IR spectra were acquired using a Perkin Elmer Spectrum spectrometer (C105146, Perkin Elmer, France) equipped with Spectrum software (Perkin Elmer, France). Spectra were collected in the range of 4000-400 cm^{-1} with 10 scans repetitions.

6.3.5.3. Swelling degree

In order to determine the swelling degree of the prepared films, samples were cut into 10×10 mm pieces, and thickness (ε_0) was measured using a micrometer caliper (Mitutoyo, USA) with an accuracy of 0.1 µm. Films were immersed on phosphate buffered saline (PBS) solution at pH 7.4 during 24h at 37 ºC and, subsequently, the thickness (ε_1) was measured [318]. The swelling volumetric degree (SW) was calculated using equation 1:

$$SW_{volumetric} = \left(\frac{\varepsilon_1 - \varepsilon_0}{\varepsilon_0}\right) \quad \text{(equation 6.1)}$$

Where $SW_{volumetric}$ corresponds to volumetric swelling, ε_0 and ε_1 correspond to the initial and final film thickness, respectively.

6.3.5.4. Thermal properties

To evaluate the thermal stability of the films prepared, thermogravimetric analyses (TGA) were performed using a TGA Q50 analyzer (TA instruments, USA). The samples were heated at a constant rate of 10 $K.min^{-1}$, from room temperature up to 873 K, under argon atmosphere. The Universal Analysis software was used to determine the onset (T_{onset}) and decomposition (T_{dec}) temperatures.

6.3.5.5. Mechanical properties

Puncture tests were carried out using a TA.XTplus texture analyzer (Stable Micro Systems, UK) equipped with a 2 mm diameter cylindrical stainless steel probe. Samples were punctured through a hole (diameter of 10 mm), at a constant speed of 1 $mm.s^{-1}$.

Puncture stress (σ, Pa) was calculated according to the following equation:

$$\sigma = \frac{F}{S_c} \quad \text{(equation 6.2)}$$

where F is the maximum force exerted by the probe (N) and S_c is the probe cross-sectional area (m^2). In order to normalize results without the influence of membrane thickness, the puncture stress values were calculated (σ_n , MPa mm^{-1}) as follows:

$$\sigma_n = \frac{\sigma}{\varepsilon_0} \quad \text{(equation 6.3)}$$

6.3.6. Biological assays

6.3.6.1. Indirect cytotoxicity assays

Cytotoxicity assays were performed by an indirect method following the ISO 10993-5:2009 guidelines for biological evaluation of medical devices [319]. In order to collect film extract, pieces of 2 cm^2 were maintained in 5mL of culture medium under controlled temperature (37 ºC) and constant stirring rate (200 rpm) for 24h. Extracts were then removed and diluted as follows: 100%, 75%, 50% and 25% using cell culture medium, for immediate use. Bj5-ta human skin fibroblasts (American Type Culture Collcetion (ATCC)) were seeded at a density of $1.25x10^5$ cells/well on a 96-well tissue culture plate and incubated for 24h, at 37°C in a humidified atmosphere with 5% CO_2 (Sanyo Electric CO, Japan). After this time, the culture medium was removed and cells were exposed to film extracts. Life control corresponded to cells simply incubated with culture medium (100% of the total volume). After 6h and 24h, cell metabolic assay was assessed by a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay, where 10% of the MTT reagent (0.5 mg/mL) dissolved in medium was added to each well, and cells were incubated at 37°C for 2 h. The MTT solution was carefully aspirated, and formazan crystals were dissolved with a DMSO/EtOH (1:1(v/v)) mixture. Absorbance was measured on a microplate reader (SpectraMax Plus, Molecular Devices, USA) at 570 nm. All the results were normalized relatively to life control, which was considered as 100% of viability. Triplicates were performed, in three independent experiments for each treatment, and the data were expressed as mean ± standard deviation. The viability results obtained for each film extract, at 6 and 24h, were analyzed using the Prism version 6.00 for Windows (GraphPad Software, USA). Where differences did exist, the source of the differences at a $p < 0.05$ of significance level was identified by all pairwise multiple comparison procedure. The Tukey's test was used for pairwise comparisons.

6.3.6.2. IL6 (Interleukin 6)-mediated skin inflammation

To determine the anti-inflammatory potential of film extracts, Bj5-ta cells were seeded at a density of $1.8x10^5$ cells/ml on a 12-well tissue culture plate for 24h before the following procedure. Then,

cells were stimulated with 5 ng/mL lipopolysaccharides (LPS) for 8h. After stimulation, culture medium was removed and inflamed cells were exposed to film extracts at 100% dilution, as well as to two EtPUAE extracts with different concentration (1 µg/ml and 10 µg/ml) and two cynaropicrin solutions with different concentration (0.5 µg/ml and 5 µg/ml) for 16h at 37°C in a humidified atmosphere with 5% CO_2. Piroxicam, a known anti-inflammatory drug, at a concentration of 10 µg/ml, was used as negative control. A human ELISA kit was used to detect the IL-6 levels in accordance with the product manual. Triplicates were performed, in three independent experiments for each treatment, and the data was expressed as mean ± standard deviation. The IL-6 results obtained for each film extract, after 8h of stimulation with LPS and after 16h exposure to film extracts were analyzed using the Prism version 6.00 for Windows (GraphPad Software, USA). Where differences did exist, the source of the differences at a $p < 0.05$ of significance level was identified by all pairwise multiple comparison procedure. The Tukey's test was used for pairwise comparisons.

6.3.6.3. Acridine orange/propidium iodide staining assay

BJ5-ta cells were seeded at $1.8x10^5$ cells/ml on a 12-well tissue culture plate and left to stabilize overnight. The next day, the same conditions as for IL-6 assays were replicated. After the respective incubations, cells were washed with PBS and incubated with a propidium iodide (PI) solution (50 µg/ml in PBS) for 15 min, after which were incubated with an acridine orange (AO) solution (50 µg/ml in PBS) for another 15 min. In all incubation periods, cells were maintained in a humidified atmosphere at 37 ºC, 5% CO_2, in the dark. Cells were then micrographed with an inverted fluorescent microscope (Olympus IX71), using FTIC and TRITC filters.

6.3.6.4. Wound healing – Scratch assay

Bj5-ta cells were seeded on an Ibidi µ-plate 24 well (Ibidi GmbH, Germany) at a density of 2.5×10^5 cells/mL for wound healing assay and according to Ibidi® instruction manual [320]. Cells were incubated in a humidified atmosphere at 37 ºC, 5% CO_2 to allow cell adhesion and the formation of a confluent monolayer. Thereafter, Ibidi culture inserts were removed, and cell monolayers with a cell-free gap of 500 µm ± 100 µm were gently washed with PBS, followed by addition of film extracts at a 100% dilution. Cells were then incubated for different time periods (0, 7 and 24h). Cells incubated in culture medium, within the same time periods, served as control. For each incubation period, the plate was placed under a phase-contrast microscope (Motic, China) equipped with a camera (Motic 2500, Motic, China) and images were acquired at 10× magnification with a computer-based microscopy imaging system (Motic Images Plus 2.0, Motic, China). Cell migration quantification was performed using Wound healing ACAS (Automated Cellular Analysis System) image analysis (Ibidi GmbH, Germany). For each image, the gap area was measured at certain time intervals and compared to the initial gap area at time t = 0. Wound closure (expressed as a percentage) was calculated from the following equation:

$$Wound\ closure\ (\%) = \frac{(W_{t0} - W_{ti})}{W_{t0}} \times 100 \qquad \text{(equation 6.4)}$$

where, W_{t0} and W_{ti} correspond to the initial and time interval measured area, respectively.

6.4. Results and discussion

Relative humidity may influence chitosan film properties. Based on this, chitosan and chitosan enriched with 1, 5 and 10% EtPUAE, were exposed to different relative humidity values, until a constant mass was achieved. The films obtained were then characterized as described below.

6.4.1. Scanning Electron Microscopy (SEM)

Chitosan films obtained by solvent evaporation method were colorless, transparent and bright. Incorporation of EtPUAE extracts at different cynaropicrin/chitosan ratios (1, 5 and 10% (w/w)), led to greenish but shiny films, as can be observed in Figure 6.1 (a1 to d1).

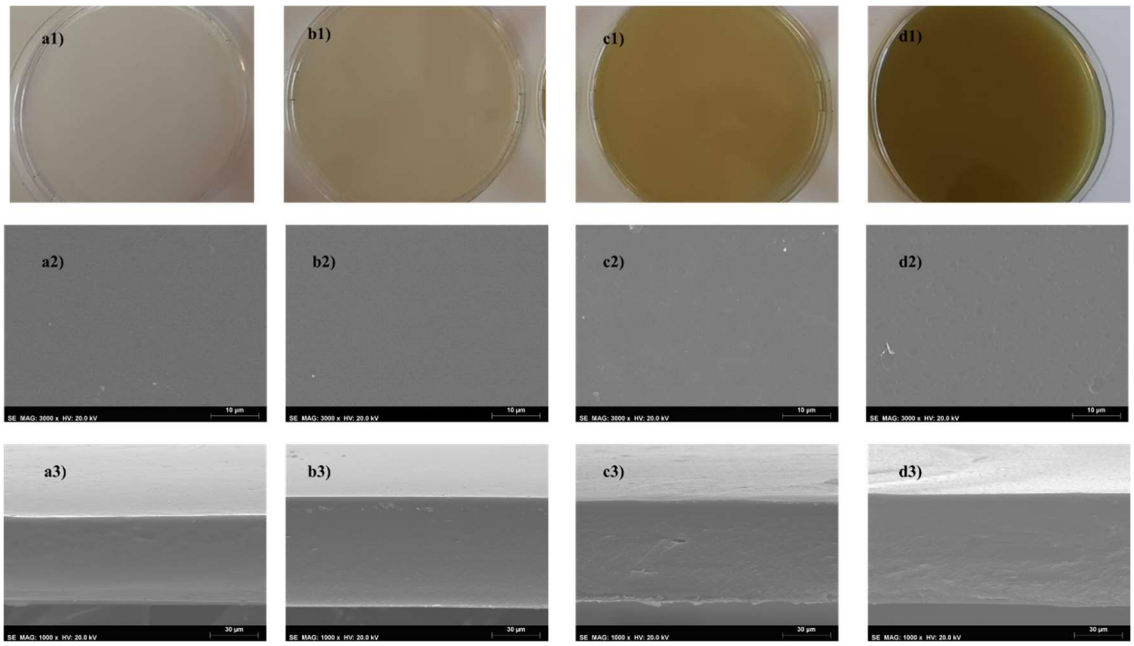


Figure 6.1. Photographs of films of chitosan (a1); chitosan + 1% EtPUAE (b1); chitosan + 5% EtPUAE (c1); and chitosan + 10% EtPUAE (d1). SEM surface images of films (amplification 3000x) of chitosan (a2); chitosan + 1% EtPUAE (b2); chitosan + 5% EtPUAE (c2); and chitosan + 10% EtPUAE (d2). SEM cross-section images of films equilibrated at RH of 57.7% (amplification 1000x) of chitosan (a3); chitosan + 1% EtPUAE (b3); chitosan + 5% EtPUAE (c3); and chitosan + 10% EtPUAE (d3).

Surface and cross section images of films obtained by SEM, at the four water activities under study, did not present significant differences between them, therefore, only surface and cross section images for an RH of 57.7% are presented in Figure 6.1.

From the surface images obtained, it is possible to observe that the films prepared are dense and homogeneous, without visible cracks or defects, and no differences detectable between the chitosan film (Figure 6.1 a2), and chitosan incorporated with EtPUAE extracts (1, 5 and 10% (w/w)) (Figures b2, c2 and d2, respectively). Regarding the cross-section images, the obtained results

show that EtPUAE extracts are well dispersed in chitosan, presenting also a dense and a homogeneous structure (figures 6.1 a3 to d3).

6.4.2. FT-IR

In order to understand the existence of possible molecular interactions between chitosan and EtPUAE extracts, a FT-IR analysis was performed to chitosan, chitosan + 1% EtPUAE, chitosan + 5% EtPUAE and chitosan + 10% EtPUAE films, at a RH of 22.6%, 57.7%, 75.3% and 83.3%. The results obtained for films conditioned at a RH of 57.7% are depicted in Figure 6.2. A similar pattern was observed for the four spectra obtained, although with some different transmittance intensities at certain peaks. Chitosan characteristic peaks corresponding to OH-stretching (3400-3300 cm^{-1}), N-H stretching in the amino group (3500-3300 cm^{-1}), C-H symmetric and asymmetric stretching (3000-2850 cm^{-1}), C=O stretching (amide I) (1650 cm^{-1}), N-H bending (amide II) (1539 cm^{-1}), C-N (amide III) (1323 cm^{-1}), asymmetric stretching of the C-O-C bridge (1153 cm^{-1}) and C-O stretching (1024 and 1066 cm^{-1}), were detected and are in accordance with previously reported results [321, 322].

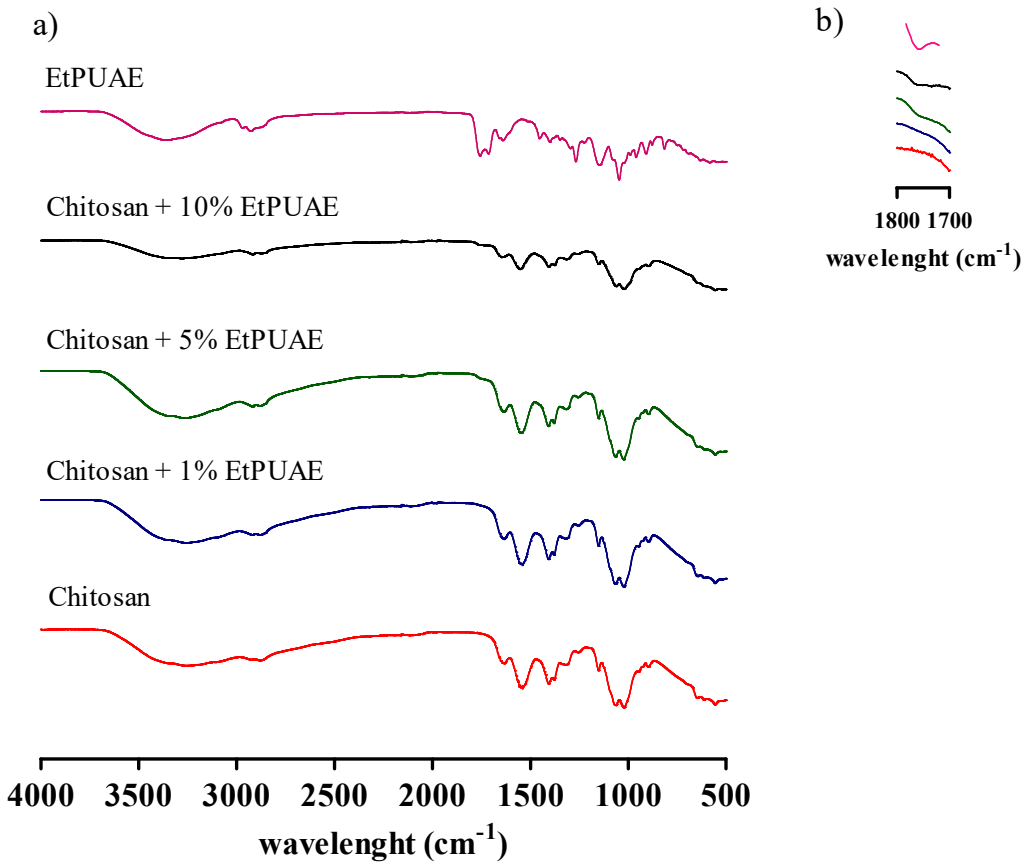


Figure 6.2. FT-IR spectra for chitosan, chitosan + 1% EtPUAE, chitosan + 5% EtPUAE, chitosan + 10% EtPUAE and EtPUAE at a RH of 57.7% where a) corresponds to full spectra and b) corresponds to detailed spectra on the sesquiterpene lactone peak wavelength zone (1700-1800 cm^{-1}).

The addition of EtPUAE extracts did not reveal significant wavelength shifts, which may indicate the inexistence of covalent bonds established between the extracts and chitosan [323]. A new peak

at 1746 cm^{-1}, characteristic of C=O stretching (δ-lactone) [324], was observed as well as an intensity increase with an increasing extract concentration in the chitosan films (Figure 6.2b). This peak, also identified on the EtPUAE spectra could be attributed to cynaropicrin, a sesquiterpene lactone identified as the major compound found on EtPUAE extracts [284].

For the chitosan film prepared with 10% (w/w) cynaropicrin, a decrease on the broad peak of -OH stretching (3400-3300 cm^{-1}) and/or -NH (1539 cm^{-1}) was observed and this may be due to possible binding interactions between cynaropicrin -OH groups (Figure 6.3a) and -OH or -NH chitosan groups (Figure 6.3b). Sun et al [323] reported the same effect with hydrogen bonds established between polyphenols and chitosan, contributing to the interaction between these two compounds.

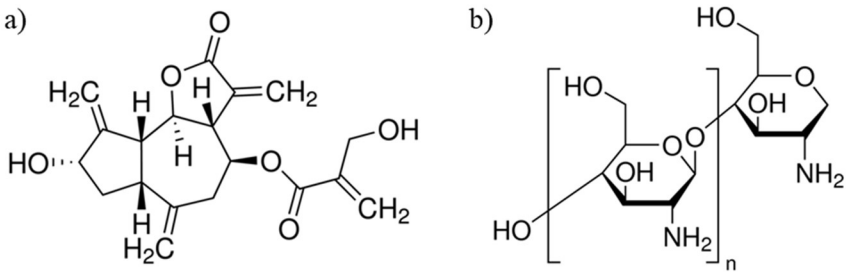


Figure 6.3. Chemical structures of cynaropicrin a) and chitosan b).

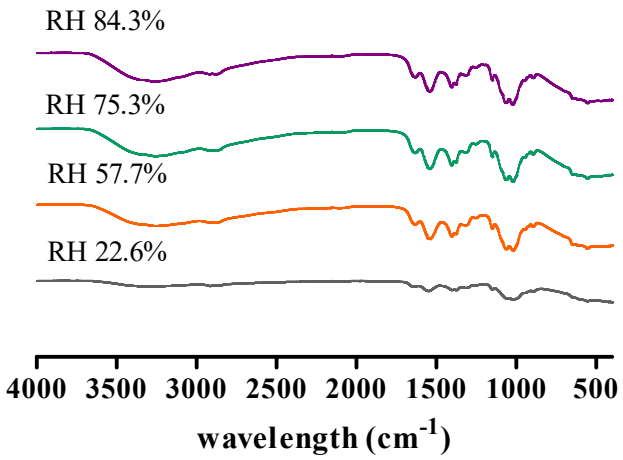


Figure 6.4. FT-IR spectra for chitosan film conditioned at different relative humidity values: 22.6%; 57.7%; 75.3% and 84.3%.

When comparing the relative humidity effect on the chitosan film spectra it was possible to observe a decrease in characteristic peaks with reducing relative humidity (Figure 6.4). A low value for the -OH vibrational band can be attributed to a lower content of water molecules, whereas the increase of intensity from 57.7% to 84.3% of RH can be associated to increasing hydrogen bonding between chitosan chains, as explained by Sanchez el al 2017 [325] .

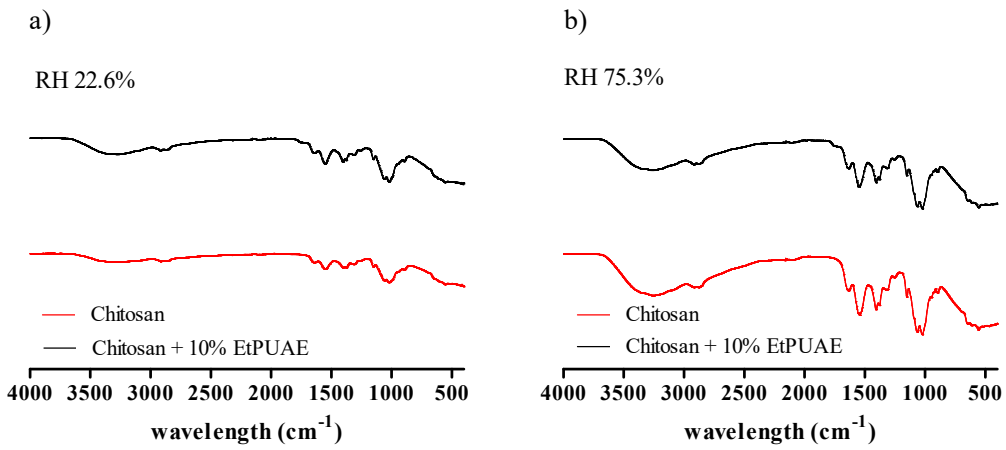


Figure 6.5. FT-IR spectra of chitosan film and chitosan + 10% EtPUAE, conditioned at a) RH 22.6% and b) RH 75.3%.

When analyzing FT-IR spectra of chitosan + 10% EtPUAE at 22.6 and 75.3% relative humidity and comparing it with neat chitosan FT-IR, respectively, Figure 6.5a) and b), it is possible to observe that, at lower RH, the film with the extract presents an -OH vibrational band more intense than the film without extract. The higher intensity observed for the chitosan + 10% EtPUAE film at low RH, comparatively to neat chitosan film, could be attributed to hydrogen bonding between extract and chitosan, which is in accordance to what was mentioned before. On the contrary, at higher RH, a similar effect cannot be observed because the higher water content overshadows the signal associated with hydrogen bonding.

6.4.3. Swelling

The creation of an optimal moist wound healing environment is critical to reduce the risk of complications on the surrounding skin and, based on this, wound dressings need to be able to absorb and retain exudates while maintaining an optimum level of moisture at the wound surface, which in turn must not spread to the surrounding skin [326]. Phosphate buffered saline (PBS) mimics, for in vitro assessment, the osmolarity and ion concentrations of the human body fluids. For this reason, PBS was selected for swelling experiments.

With respect to the swelling effect upon the different $w_{cyn}/w_{chitosan}$ ratios tested, it was possible to observe a general decrease of the film swelling capacity, with increasing EtPUAE extract concentration (Table 6.2). The presence of hydrophobic compounds (e.g. cynaropicrin) reduces polymer hydrophilicity, decreasing its affinity to PBS, leading to a lower swelling ability, as observed by several authors during the addition of lipidic fractions into chitosan films [322, 327, 328].

An increase of chitosan volumetric swelling was observed with an increase of the relative humidity, from 134% to 945% ($p < 0.05$) (Table 6.2).

As explained by some authors, a high relative humidity increases hydrophilic polymers moisture content, opening new binding sites for water, increasing its ability to PBS sorption, as observed for

chitosan films [329]. The incorporation of EtPUAE, due to its hydrophobic character, reverses this increase, leading to the lowest volumetric swelling effect. Considering an optimum exudate control on wound site, higher EtPUAE content should be considered for light exudate wounds. Nevertheless, all films presented higher swelling degree comparing to the comercially available dressing, Tegaderm™ (9% after 48h) [190].

Table 6.2. Volumetric swelling values obtained after 24 h immersion in a PBS solution, for chitosan, chitosan +1% EtPUAE, chitosan + 5% EtPUAE, chitosan + 10% EtPUAE, for films prepared under four different relative humidities of 22.6, 57.7, 75.3 and 84.3%.

	Chitosan	Chitosan + 1% EtPUAE	Chitosan + 5% EtPUAE	Chitosan + 10% EtPUAE
RH = 22.6%	134 ± 5^{a}	151 ± 3^{a}	103 ± 3^{a}	76 ± 4^{a}
RH = 57.7%	363 ± 25^{b}	115 ± 9^{b}	90 ± 6^{b}	72 ± 12^{a}
RH =75.3%	453 ± 31^{c}	$94 \pm 14^{b,c}$	62 ± 9^{c}	46 ± 5^{b}
RH =84.3%	945 ± 35^{d}	77 ± 8^{c}	66 ± 4^{c}	45 ± 6^{b}

Average ± standard deviation estimated from three extracts analyzed in triplicate.
Values with different superscripts, within the same column, are statistically different ($p < 0.05$)

6.4.4. Thermogravimetric analysis (TGA)

Thermogravimetric results for films of chitosan and chitosan loaded with EtPUAE are represented in Figure 6.6. For the chitosan film, a first mass loss was observed between 307 K and 428 K, corresponding to evaporation of water molecules present in the films [330]. Thermal decomposition of the chitosan film occurs between 400 K and 650K, due to denaturation of the chitosan polymeric structure, which is in accordance with the literature [330-332].

In Table 6.3 and Figure 6.6, it is possible to observe that, although T_{onset} decreases with the increase of cynaropicrin concentration in films, such decrease is not significant, for the same relative humidity and for the different chitosan – based films under study. Exception made for the RH of 22.6%, where a higher percentage weight loss was observed for the chitosan film, which could be attributed to the lower moisture content, as observed from the swelling results.

Table 6.3. Onset temperature (Tonset (K)) and decomposition temperature (Tdec (K)) for the different films under study, chitosan, chitosan + 1% EtPUAE, chitosan + 5% EtPUAE and chitosan + 10% EtPUAE and for the relative humidity values tested: 22.6, 57.7, 75.3 and 84.3 %.

	Chitosan		Chitosan + 1% EtPUAE		Chitosan + 5% EtPUAE		Chitosan + 10% EtPUAE	
	T_{onset} (K)	T_{dec} (K)	T_{onset} (K)	T_{dec} (K)	T_{onset} (K)	T_{dec} (K)	T_{onset} (K)	T_{dec} (K)
RH=22.6%	431	538	428	522	426	532	421	542
RH=57.7%	435	647	425	546	428	546	428	546
RH=75.3%	426	546	419	546	422	546	421	544
RH=84.3%	438	542	438	545	435	542	436	547

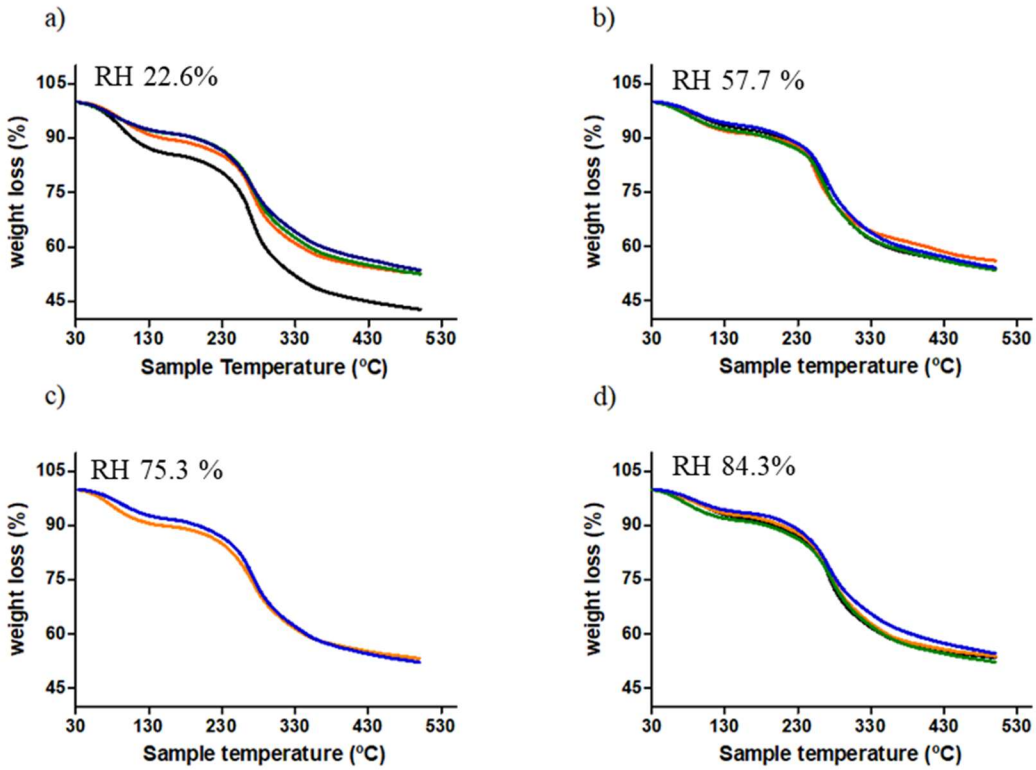


Figure 6.6. TGA results expressed in terms of sample weight loss (%) as a function of sample temperature (ºC) for chitosan (black), chitosan + 1% EtPUAE (red), chitosan + 5% EtPUAE (green) and chitosan + 10% EtPUAE (blue), conditioned at a) RH 22.6%, b) RH 57.7%, c) RH 75.3%, d) RH 84.3%.

The results obtained show that the thermal stability of chitosan films is not affected by the incorporation of EtPUAE extracts and relative humidity. Considering the final application as wound dressing, the TGA analysis revealed that, at 37 ºC, no weight loss was observed for the chitosan-based films, supporting their suitability for skin wound application.

6.4.5. Mechanical properties

The values of tension under puncture are represented on Table 6.4. The results show that, for the same relative humidity, the incorporation of EtPUAE extracts promotes a decrease of the films tension upon rupture, although it is not observed a linear and statistically different decrease with the increase of the EtPUAE film content ($p < 0.05$). As stress represents the maximum strength that films can support before breaking, the results indicate that films produced with the EtPUAE extract incorporation present a lower mechanical resistance when compared with that produced only with chitosan. A similar behavior was observed by other authors, when adding carvacrol, α-tocopherol or apple polyphenols to a chitosan matrix [323, 333, 334]. It was proposed that the addition of hydrophobic compounds to the hydrophilic chitosan medium, induces the development of structural discontinuities in the highly packed chitosan matrix, lowering its ability to resist to mechanical solicitations [335]. The same behavior is envisaged to occur in the present work, considering the EtPUAE hydrophobic behavior and the molecular interactions between EtPUAE compounds and the chitosan polymer, which may weaken the polymer chain aggregation forces.

Furthermore, films with the same extract content show a substantial decrease of puncture stress with an increase of the relative humidity. The lowest value (60.11±5.66 MPa.mm^{-1}) was observed for the highest relative humidity (84.3%) and the highest EtPUAE content (10%). This fact is attributed to the plasticizer effect of sorbed water that reduces polymer-polymer interactions, lowering the films mechanical strength. However, the lower tensile strength obtained, did not prevent their use on skin healing since that according to some authors, the tensile strength of human skin tissue, varies between 5 and 30 MPa [336] although some authors have suggested that pre-stresses may be as great as 1 MPa [337].

Table 6.4. Tensile strength (MPa/mm) and elongation at break (%) for chitosan, chitosan + 1% EtPUAE, chitosan + 5% EtPUAE and chitosan + 10% EtPUAE for the relative humidity tested, 22.6, 57.7, 75.3 and 84.3%.

RH (%)	Tension (MPa/mm)			
	Chitosan	Chitosan + 1% EtPUAE	Chitosan + 5% EtPUAE	Chitosan + 10% EtPUAE
22.6	215.55±19.04^{a}	139.15 ± 1.92^{b}	124.8±0.44^{c}	114.75±0.26^{d}
57.7	137.90±0.83^{a}	111.61±4.03^{b}	102.43±7.99^{b}	106.85± 2.25^{b}
75.3	115.23±4.37^{a}	110.20±3.38^{a}	79.91±5.48^{b}	78.34±11.59^{b}
84.3	88.59±1.05^{a}	83.74± 0.55^{a}	81.09±8.94^{a}	60.11±5.66^{b}

Average ± standard deviation estimated from three extracts analyzed in triplicate.
Values with different superscripts, within the same column, are statistically different ($p < 0.05$)

6.4.6. Biological activity

6.4.6.1. Cytotoxicity

Biocompatibility, including low or absence of cytotoxicity are key factors for a material to be considered suitable for use as medical device. According to ISO 10993-5:2009 guidelines for biological evaluation of medical devices, a reduction on cell viability higher than 30% is considered to present a cytotoxic effect [319]. Indirect cytotoxicity assays for chitosan films, with extracts at different dilution rates, were performed with telomerase-immortalized human normal skin fibroblasts (Bj5-ta cell line) for incubation periods of 6 and 24h, as presented in Table 6.5. According to the obtained results , after 6h of exposure, the chitosan film led to a cell viability of 98.4 ± 4.6%, and the chitosan + 1% EtPUAE film resulted in a cell viability of 95.7 ± 5.0%, with no statistical difference between them ($p < 0.05$). Although leading to a lower cell viability, according to the ISO 10993-5:2009 guidelines, the chitosan + 5% EtPUAE film (75.51 ± 5.67) was not considered cytotoxic. On the other hand, with a cell viability of 51.09 ± 5.03%, the chitosan + 10% EtPUAE film was considered cytotoxic. After 24h, no statistical difference was observed between the values for the non-cytotoxic films (neat chitosan and chitosan + 1% EtPUAE).

The IC_{50} values at 6 and 24h for the EtPUAE extract and pure cynaropicrin was determined. In addition, cynaropicrin content in the film extracts used for the cytotoxicity experiments, was quantified. Analysis of the cynaropicrin content, shows that films with incorporation of 1 or 5 % (w/w) EtPUAE extract contain respectively 0.177 and 2.96 µg/mL of cynaropicrin, a concentration below

its IC_{50} (15.92 ± 1.45 µg/mL). Regarding the film of chitosan + 10% EtPUAE, at 6h of incubation, a significant cell viability reduction was observed (51.09 ± 5.03%). The final cynaropicrin concentration of film extract was 17.73 µg/mL (chitosan + 10% EtPUAE film) indicating that the observed cytotoxicity is most probably directly correlated with the cynaropicrin concentration in the film.

Table 6.5. Bj5-ta cell viability (%) after 6 and 24h of incubation for films of chitosan, chitosan + 1% EtPUAE, chitosan + 5% EtPUAE and chitosan + 10% EtPUAE, as well as cynaropicrin concentration in each film extract. IC_{50} (µg/mL) for the Bj5-ta cell line, after 6 and 24h of incubation, for EtPUAE and cynaropicrin.

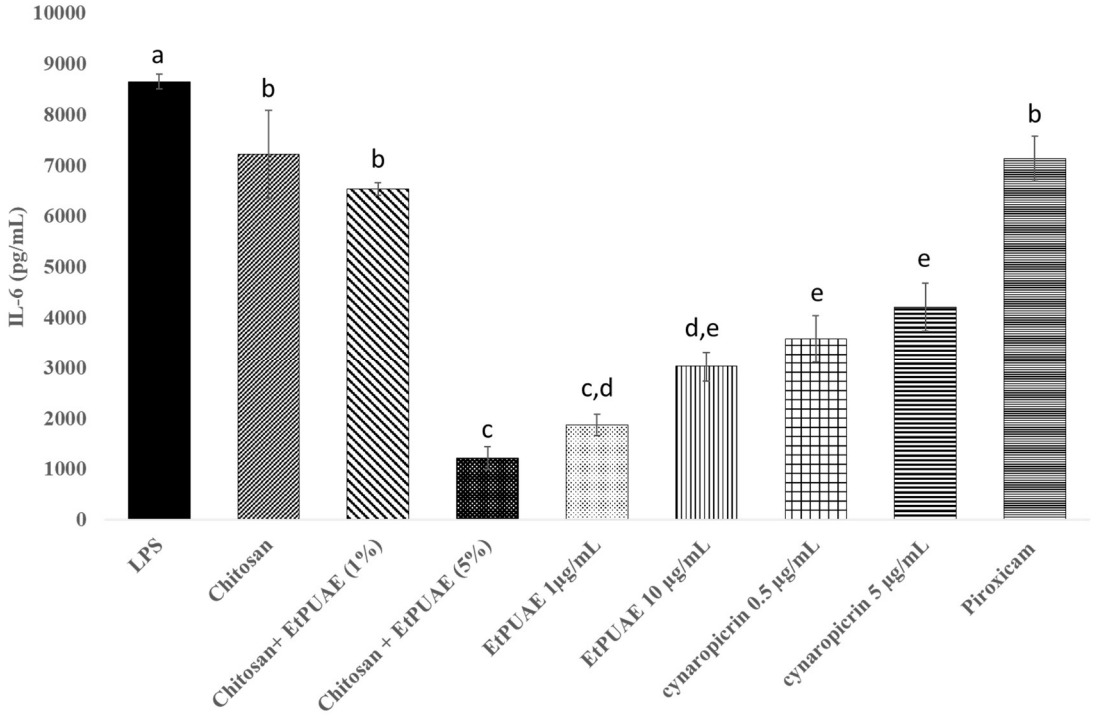
Film	Cell viability (%)		Cyn film extract (µg/mL)
	6h	24h	
Chitosan	98.4 ± 4.6^{a}	95.8 ± 7.1^{a}	-
Chitosan + 1% EtPUAE	95.7 ± 5.0^{a}	88.5 ± 8.9^{a,b}	0.177
Chitosan + 5% EtPUAE	75.6 ± 5.7^{b}	81.4 ± 8.9^{b}	2.96
Chitosan + 10% EtPUAE	51.1 ± 5.0^{c}	34.1 ± 2.7^{c}	17.73
	IC50 (µg/mL)		
EtPUAE	36.1 ± 1.6	24.1 ± 1.1	-
Cynaropicrin	15.9 ± 1.4	9.7 ± 1.0	-

Average ± standard deviation estimated from three extracts analyzed in triplicate.
Values with different superscripts, within the same column, are statistically different ($p < 0.05$)

6.4.6.2. IL6 (Interleukin 6)-mediated skin inflammation

Continuous inflammation is a major cause of chronic wounds, and the expression modulation of pro-inflammatory interleukins is a suitable way to stop the inflammatory process, reducing epithelization, thus promoting a normal wound healing process [338]. IL-6 is one of the key pro-inflammatory factors produced by dermal fibroblasts in response to certain stimuli [339]. In this work, dermal fibroblasts (Bj5-ta) were stimulated with liposaccharide (LPS) and after this activation, which induces the IL-6 production, cells were exposed to the chitosan-based films under study. As controls, the EtPUAE extract and a cynaropicrin solution were directly applied to the stimulated Bj5-ta cells.

Interestingly, the IL-6 levels were reduced on stimulated Bj5-ta cells exposed to the film extracts (neat chitosan, chitosan+1% EtPUAE and chitosan+5% EtPUAE) (Figure 6.7). A detailed analysis of results shows that, for the neat chitosan film, a reduction of IL-6 expression by LPS-stimulated fibroblasts was achieved, without statistical difference, in comparison with 10 ng/mL piroxicam. Incorporation of EtPUAE into chitosan films seems to promote an anti-inflammatory effect as expressed by reduction of IL-6 levels, although without statistical difference comparing to neat chitosan ($p > 0.05$). The strongest effect was observed for the chitosan + 5% EtPUAE film, with a reduction of ≈ 86% and 83% in the IL-6 expression by skin fibroblasts, comparatively to LPS and the neat chitosan film ($p < 0.05$), which is extremely promising for regulation of continuous inflammation in chronic wounds.



Average ± standard deviation estimated from three extracts analyzed in triplicate.
Values with different superscripts, within the same column, are statistically different ($p < 0.05$)

Figure 6.7. ELISA quantification of IL-6 produced by LPS-stimulated BJ-5ta cells after exposure to films of chitosan, chitosan + 1 % EtPUAE, chitosan + 5% EtPUAE, and EtPUAE extracts at 1 and 10 µg/mL and a solution of cynaropicrin at 0.5 and 5 µg/mL. Piroxicam was used as a negative control at 10ng/mL. Levels of IL-6 were measured after 8 h of incubation with the samples.

Further, the effect of the EtPUAE extract and the cynaropicrin solution was also evaluated, taking into account the cynaropicrin levels quantified in chitosan-based films (Table 6.5). With a cynaropicrin content of 51.7 mg/g extract, a higher reduction of IL-6 levels was observed with the EtPUAE extract, when compared to the pure cynaropicrin solution (in the same concentration range). Although also effective, the EtPUAE extract at 10 µg/ml has a lower capacity to reduce IL-6 levels in stimulated cells when compared to the EtPUAE 1 µg/ml ($p < 0.05$), which may be related to a slight increase in the material's toxicity with the higher extract load. Although EtPUAE, as well as cynaropicrin, can reduce IL-6 levels according to the results presented in Figure 6.7, there are no differences in regard to this cytokine quantification for cells incubated with EtPUAE extract (10 µg/ml) or cynaropicrin (0.5 and 5 µg/ml, 1.44 and 14.4 µM, respectively) ($p > 0.05$). These results support a more potent anti-inflammatory effect for the EtPUAE extract, comparatively to cynaropicrin, underlying an interesting synergetic effect for the extract.

Taking into account the cytoxicity associated to cynaropicrin at 5 µg/mL (14.4µM) (Table 6.5), a dual staining with Acridine Orange (AO) and Propidium Iodide (PI) was performed to distinguish viable cells from apoptotic/necrotic cells. Vander Berghe et al. [340] observed that necrosis is induced by several stimuli, such as Tumor Necrosis Factor (TNF), anti-Fas (an apoptotic mediator) or double-stranded RNA, promote the secretion of the pro-inflammatory cytokine IL-6. According to our results, Table 6.6 and Figure 6.8, the EtPUAE extract at 10 µg/mL, and the cynaropicrin solution at 5µg/mL, induce a slight increase in cell death, as more PI (red) fluorescence is visible,

comparatively to the lower tested concentrations. This could be related to necrosis promoted by high levels of the EtPUAE extract and cynaropicrin, mediated by increased levels of IL-6, as described by Vander Berghe et al.

Regarding the chitosan-based films, a very marginal reduction in the number of viable cells (green fluorescence) was observed for the chitosan + 5% EtPUAE film, which may also be related to extract-related toxicity.

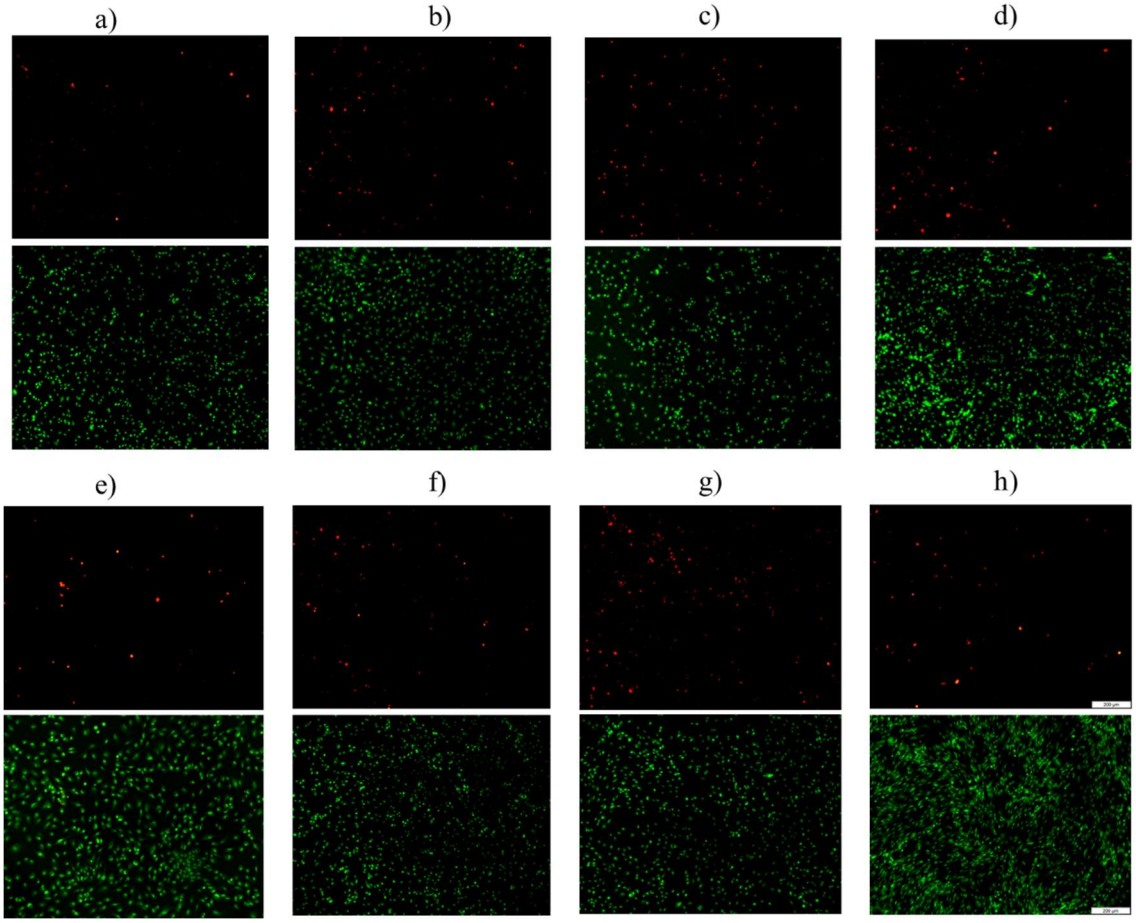


Figure 6.8. Acridine orange (AO, green) and propidium iodide (PI, red) double staining fluorescent micrographs of LPS stimulated Bj5-ta cells after 16h of exposure to: (a) chitosan film, (b) chitosan + 1% EtPUAE film, (c) chitosan + 5% EtPUAE film, (d.) EtPUAE 1µg/mL, (e) EtPUAE 10µg/mL (f) positive control (LPS) (g) negative control (piroxicam) (h) unstimulated cells (life control). The images were captured in multiple times with a x20 magnification.

Although this is the first time that cynaropicrin and EtPUAE extracts are tested in LPS-stimulated Bj5-ta fibroblasts, cynaropicrin's anti-inflammatory potential has been previously monitored by IL-6 expression downregulation, in human keratinocytes. According to Takei et al, cynaropicrin at 1 µM appeared to inhibit IL-6 and TNF-α upregulation in UVB-treated normal human epidermal keratinocytes [341]. The results presented here show that EtPUAE extracts and loaded chitosan films reveal a similar IL-6-mediated anti-inflammatory activity in skin fibroblasts, which supports their applicability for chronic wound management.

Table 6.6. Cell viability for Bj5-ta LPS stimulated cells after 16h of exposure to films of chitosan, chitosan + 1% EtPUAE, chitosan + 5% EtPUAE and EtPUAE at 1 and 10 µg/mL, calculated from AO+PI assay.

	Cell viability (%)
Chitosan	96.0 ± 0.2
Chitosan + 1% EtPUAE	95.0 ± 2.0
Chitosan + 5% EtPUAE	90.0 ± 2.2
EtPUAE 1ug/mL	96.0 ± 0.7
EtPUAE 10ug/mL	90.0 ± 4.0
LPS	95.0 ± 1.3
Piroxicam	79.0 ± 4.1

6.4.6.3. Scratch assay

Although a stronger reduction in IL-6 levels of LPS-stimulated fibroblasts was observed for the film of chitosan + 5% EtPUAE, a decrease in cell viability was also evident, which indicates a potential toxicity issue. Therefore, wound healing assays were only performed for films of chitosan and of chitosan + 1% EtPUAE.

Fibroblasts are responsible for most collagen and elastin synthesis as well as organization of the extracellular matrix (ECM) components [342]. Therefore, normal skin fibroblasts were selected for this study due to their need for determination of wound closure. Analysis of results reported on Table 6.7 and Figure 6.9, shows that, after 7 h of incubation, films with extract did not affect fibroblasts migration with statistical significance ($p > 0.05$), comparatively to the sample control. On the other hand, after 24h of incubation, a more effective wound closure was observed for the film with 1% EtPUAE ($p < 0.05$), which suggests that a concentration of 0.177 µg/mL cynaropicrin can promote a faster wound healing.

Table 6.7. Bj5-ta fibroblast wound closure rate after 7h and 24h of incubation with films of chitosan and chitosan + 1 % EtPUAE.

	7h	24h
Control	87±3%^{a}	22±6%^{a}
Chitosan	83±9%^{a}	28±8%^{a}
Chitosan + 1% EtPUAE	84±6%^{a}	15±9%^{b}

Average ± standard deviation estimated from three extracts analyzed in triplicate.
Values with different superscripts, within the same column, are statistically different ($p < 0.05$)

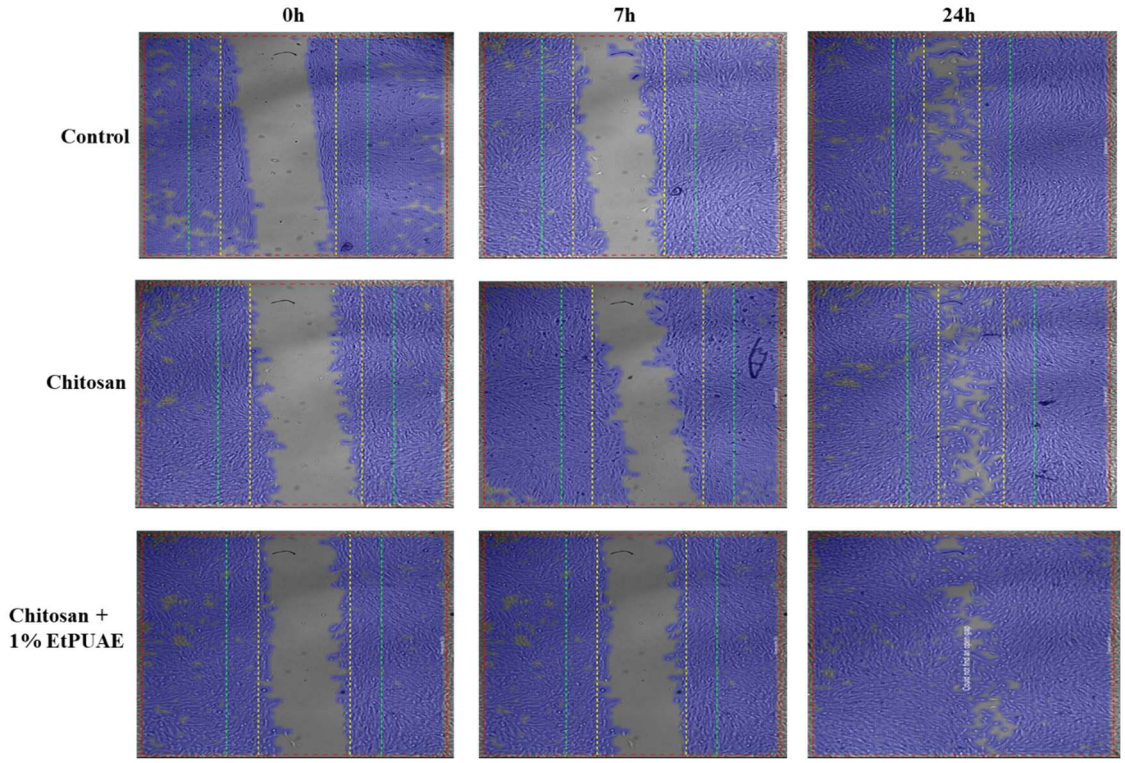


Figure 6.9. Microscopical images representing the in vitro scratch assay of chitosan based films: Bj5-ta cells were incubated in the presence and absence of chitosan based films and imag-es were captured at 7h and 24h. The boundaries of the scratched wounds were determined by the yellow lines.

6.5. Conclusions

In this work chitosan and chitosan films loaded with different EtPUAE extracts were prepared, conditioned at different relative humidity values and characterized in terms of structure, swelling, thermal and mechanical properties. In addition, preliminary assessment of the biological properties (cytotoxicity, IL-6 mediated skin inflammation and wound healing) were performed in order to evaluate the prospective use of the films developed as wound dressing materials.

SEM analysis of chitosan and chitosan-based films produced, revealed a homogeneous and dense structure, although after EtPUAE loading, to the naked eye, green and shiny films were obtained, in opposition to clear and translucent neat chitosan films. A decrease of the volumetric swelling capacity was observed with the EtPUAE loading, attributed to the extract hydrophobic character, which could be advantageous for wound dressings where an excessive fluid sorption is not desired. A decrease of the swelling effect was observed with the increase of relative humidity for neat the chitosan film, with the reverse effect observed for chitosan + EtPUAE films.

Chitosan-based FT-IR analysis did not reveal the presence of new covalent bonds established between chitosan and the incorporated extract, although an increase of -OH bands intensity was observed, which seems to indicate the presence of hydrogen bonding between EtPUAE and chitosan. Although no effects were apparent on the chitosan thermal stability, a decrease of the tensile strength with EtPUAE loading was observed. However, an increase of the tensile stress was

obtained with the increase of relative humidity, attributed to a water plasticizer effect, with values obtained in accordance with skin tensile strength.

Biological studies show that, after 24h of exposure, leading to a cell viability of 34.1 ± 2.7 % of Bj5-ta fibroblasts, the film of chitosan + 10% EtPUAE was considered to be cytotoxic, which was related with its cynaropicrin content. On the analysis of their anti-inflammatory activity, regarding the regulation of IL-6 levels on LPS-stimulated skin fibroblasts, both films of chitosan and chitosan + 1% EtPUAE show the same effect as the one obtained with piroxicam, with no statistical difference being observed. Nevertheless, the film of chitosan + 5% EtPUAE led to a significant reduction in the IL-6 levels comparatively to the LPS stimulated cells and with no treatment, lower than what is achieved with the commercial anti-inflammatory compound (at 10 ng/mL), indicating its potential for chronic wound management. Besides that, a higher closure rate was achieved with the film of chitosan + 1 % EtPUAE when compared to either the chitosan film, and the control.

Produced with naturally sourced materials, such as chitosan and *Cynara cardunculus* leaves this work opens up new perspectives for the use of chitosan films doped with EtPUAE extracts from *Cynara cardunculus* leaves for wound dressing materials, specifically for chronic wounds trapped in continuous inflammation.

7

Conclusions and Future Perspectives

This thesis was focused on the development of natural bioactive extracts, followed by the study of their potential application in wound dressings for chronic wounds. The achievements and conclusions may be divided in two major sections, as defined by the research strategy: a) Optimization of cynaropicrin extraction methodologies from *C. cardunculus* leaves and extract fractionation by membrane processes; b) Development of chitosan-based films loaded with cynaropicrin and assessment of their biological anti-inflammatory properties.

7.1. Optimization of cynaropicrin extraction methodologies from *Cynara cardunculus* leaves and extract fractionation by membrane processing

Due to its high availability in *Cynara cardunculus* leaves and its biological potential, namely anti-inflammatory, cynaropicrin was selected as the sesquiterpene lactone target bioactive compound. A new approach for the production of cynaropicrin enriched extracts from *Cynara cardunculus* leaves was developed with the purpose of reaching two sequential objectives: i) the development of optimized extraction methodologies for production of cynaropicrin enriched extracts; ii) increase of produced extract biological potential by fractionation with membrane technology.

Cynaropicrin extraction optimization was performed by an initial comparison between conventional and non-conventional extraction methodologies upon cynaropicrin from *Cynara cardunculus* leaves. The influence of the extraction solvent upon cynaropicrin extraction yield was also studied. Batch solid-liquid as conventional and microwave assisted extraction, pressurized liquid extraction and ultrasound assisted extraction as non-conventional extraction methodologies were selected. The solvents studied were ethanol, ethyl acetate, ethanol/water (40/60) and water. Dichloromethane and Soxhlet extraction were applied as solvent and extraction control method, respectively. Applied for the first time for SL, the Hansen solubility parameters were used, renewing this model importance on general analyte solubility prediction when using conventional extraction methodologies. However, the model still presents some limitations that may be attributed to the

plant matrix complexity. The application of non-conventional extraction methodologies, using biocompatible solvents, allowed for an increase of cynaropicrin extraction yield, with UAE combined with ethanol as extraction solvent, presenting the higher extraction yield (55.00 ± 2.92 mg/g DW) as well as the lowest energy consumption (0.027 kWh/$g_{cynaropicrin}$). On the other hand, MAE extraction and water as extraction solvent, showed the lowest extraction efficiency (30.82 ± 1.15 mg/g DW) mainly due to an overheating effect and consequent degradation of cynaropicrin.

After selection of the solvent and extraction methodology, ultrasound extraction optimization was performed. First by the study of pulse effect at different duty cycles, where a duty cycle of 25% presented a higher extraction kinetic rate and a 45% energy reduction expressed as kWh/$g_{cynaropicrin,}$ comparatively to continuous mode. The extraction methodology was further optimized by a Response Surface Methodology, with 3 levels for the correlation of independent variables with the extraction yield (mg of cynaropicrin/g DW) and cynaropicrin concentration (mg cynaropicrin /g extract). The effects of S/L ratio, amplitude and temperature were evaluated regarding cynaropicrin extraction yield, as well as the interactive effect of S/L ratio and amplitude, and amplitude with temperature. The individual effects on cynaropicrin concentration expressed in mg/g extract were obtained for the S/L ratio and amplitude, as well as the interactive effect of these variables. Optimal conditions were found for a solid/liquid ratio of 1/27, amplitude of 67% and temperature of 44 °C, with obtained experimental values of 23.90 ± 0.14 mg/g DW and 192.51 ± 6.96 mg/g extract for extraction yield and cynaropicrin concentration, respectively. The predicted values fit well the experimental ones, with a 95% confidence level showing the accuracy of the model.

The chemical analysis of the obtained optimized extracts by HPLC, indicate the presence of low molecular weight compounds, such as carbohydrates, that may promote a decrease of the extract biological potential. In order to remove these compounds from the final extract, a membrane fractionation process was applied to the extract. A Duramem® 200 membrane was selected for cynaropicrin recovery and removal of carbohydrates, operated in a diananofiltration mode followed by nanofiltration, for additional concentration of cynaropicrin. A removal of 93.0% of glucose and 95.6% of fructose, with a cynaropicrin maximum loss of 13.9% was obtained, after 5 diavolumes and a concentration factor of 2 was achieved with the final nanofiltration operation. In order to evaluate the biological potential benefit of carbohydrates removal, initial and fractionated extracts viability was tested using a BJ5-ta human normal fibroblast cell line. Cell viability analysis indicated that cynaropicrin is probably the major active compound responsible for the extract activity on the Bj5-ta cell line. From that, a comparison between predicted and experimental IC_{50} for initial and fractionated extracts considering a cynaropicrin standard IC_{50} (10.3 ± 1.3 $\mu g_{cynaropicrin}$/mL), as well as the cynaropicrin content in the extracts (439 and 517 mg/$g_{dry\ extract}$ for initial and fractionated extracts, respectively), was performed. The experimental results obtained, show a decrease of IC_{50} for the fractionated extract (from 29.9 ± 4.2 to 21.4 ± 2.2 $\mu g_{extract}$/mL) on contrary to the predicted ones, where no statistical difference was observed (23.5 ± 1.2 and 19.9 ± 1.2 $\mu g_{extract}$/mL for initial and fractionated extracts, respectively). The lower biological activity of the initial extract was mainly attributed to the presence of low biological potential compounds, such as sugars, that could be trapping cynaropicrin biological activity. Considering the integrated production of cynaropicrin

enriched extracts from *Cynara cardunculus* leaves, an economical evaluation was assessed for an annual production of a cynaropicrin enriched extract of 520 kg/year, assuring a pay-back period of 4.58 years with extract selling price as key parameter, to which the pay-back period is more sensitive.

7.2. Development of chitosan-based films loaded with cynaropicrin and assessment of their biological anti-inflammatory properties.

Application of the produced extract in chitosan-based films, may cause positive or negative changes on the physico-chemical properties of chitosan films. In order to develop chitosan films incorporating cynaropicrin enriched extracts (EtPUAE), solvent evaporation method was applied and the resultant films were characterized. The physico-chemical film properties revealed that extract incorporation has an effect on the volumetric swelling capacity and the mechanical film properties, leading to a lower fluid absorption capacity and more fragile films, although not preventing their use on skin. However, no effect was observed on the films thermal properties, as well on their dense structure observed by SEM analysis. The biological potential of chitosan-based films show that chitosan + 1% EtPUAE and chitosan + 5% EtPUAE did not present a cytotoxic effect on Bj5-ta human skin fibroblasts cells. Nevertheless, a positive effect on skin inflammation, with an 86% reduction of interleukin IL-6, was achieved when Bj5-ta Liposaccharides (LPS) stimulated cells were exposed to the chitosan + 5% EtPUAE film extract.

The application of cynaropicrin enriched extract in chitosan films, for wound dressing applications, presented an augmented anti-inflammatory capacity, disclosing its potential as wound dressing for chronic wounds. The alliance between the use of low cost biomass, economic viable production process and application of cost effective wound dressings reveals to be a viable alternative that should be considered.

7.3. Future work and prospectives

The present thesis proposes the development of an integrated process for production of bioactive plant-derived extracts, with a focus on sesquiterpene lactones as bioactive group, followed by its application on chitosan wound dressing for chronic wounds treatment. However, the work developed can further extended and three possible lines for future work are described as follow.

a) Extraction industrial application

Several research groups and food industries have shown that ultrasound assisted extraction can be scale-up and applied at industrial scale. However, some key parameters should be considered for optimization of ultrasound assisted extraction (UAE) scale-up.

a.1) Absorbed Energy Density (AED) and Absorbed Power Density (APD) are UAE parameters that indicate the heating energy and power experienced by a unit volume of extraction. Obtained by calorimetric experiments, AED and APD should be obtained firstly at lab scale and used as guidelines for industrial scale-up.

a.2) Considering ultrasound extractors equipment limitations, as well as energy costs, scale-up optimization should also consider the application of continuous flow instead of batch processing.

b) Wound dressing

b.1) As wound dressing, further physico-chemical characterization of chitosan based films developed on this thesis should be performed. Among these characterizations, water vapor permeability and oxygen and carbon dioxide permeabilities should be assessed. Nevertheless, further biological experiments should be also performed, namely, *in vitro* degradation and *ex-vivo* skin permeation.

b.2) Chitosan based films properties could be improved by addition of crosslinking agents, as well as plasticizers, with sodium tripolyphosphate and glycerol, as respectively examples. Optimization should be performed regarding the improvement of mechanical properties or barrier transport as well as kinetic of transdermal drug release.

b.3.) For a better comparison and understanding of the novelty and interest of the produced films, commercially available films for chronic wounds should be tested and characterized (e.g. silver releasing dressings).

c) Biological potential of sesquiterpene lactones (*Cynara cardunculus*)

c.1) Besides cynaropicrin, several studies have also identified the presence of other sesquiterpene lactones in *Cynara cardunculus* leaves, such as grosheimin, deacylcynaropicrin and/or aguerin, although in lower quantities. From that, further detailed analysis of the ethanolic ultrasound assisted extract should be performed in order to identify the presence of those sesquiterpene lactones and performing their quantification. Synthetic formulations should be performed considering the SL content in extracts, using crescent complexity formulations (from a single sesquiterpene lactone towards the final four) in order to identify possible synergies, as well if some of the sesquiterpene lactones is responsible for the majority of the extract anti-inflammatory bioactivity.

c.2) Regarding the anti-inflammatory activity of the extract, besides the IL-6 pro-inflammatory cytokine, evaluated in this thesis, the extract potential over other inflammation mediators, should be assessed, namely α-TNF, IL-8, IL-1β and growth factors such as, transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), transforming growth factor-α (TGFα), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), and epidermal growth factor (EGF).

References

[1] T. Khushnud, S.A. Mousa, Potential Role of Naturally Derived Polyphenols and Their Nanotechnology Delivery in Cancer, Molecular Biotechnology, 55 (2013) 78-86 https://doi.org/10.1007/s12033-012-9623-7.

[2] B.V. Bonifácio, P.B.d. Silva, M.A.D.S. Ramos, K.M.S. Negri, T.M. Bauab, M. Chorilli, Nanotechnology-based drug delivery systems and herbal medicines: a review, International Journal of Nanomedicine, 9 (2014) 1-15 https://doi.org/10.2147/ijn.s52634.

[3] R. Watkins, L. Wu, C. Zhang, R.M. Davis, B. Xu, Natural product-based nanomedicine: recent advances and issues, International Journal of Nanomedicine, 10 (2015) 6055-6074 https://doi.org/10.2147/ijn.s92162.

[4] A.G. Atanasov, B. Waltenberger, E.-M. Pferschy-Wenzig, T. Linder, C. Wawrosch, P. Uhrin, V. Temml, L. Wang, S. Schwaiger, E.H. Heiss, J.M. Rollinger, D. Schuster, J.M. Breuss, V. Bochkov, M.D. Mihovilovic, B. Kopp, R. Bauer, V.M. Dirsch, H. Stuppner, Discovery and resupply of pharmacologically active plant-derived natural products: A review, Biotechnology Advances, 33 (2015) 1582-1614 https://doi.org/10.1016/j.biotechadv.2015.08.001.

[5] K. Saito, F. Matsuda, Metabolomics for Functional Genomics, Systems Biology, and Biotechnology, Annual Review of Plant Biology, 61 (2010) 463-489 https://doi.org/10.1146/annurev.arplant.043008.092035.

[6] U. Roessner, D.M. Beckles, Metabolite Measurements, in: J. Schwender (Ed.) Plant Metabolic Networks, Springer New York, New York, NY, 2009, pp. 39-69 ISBN: 978-0-387-78745-9.

[7] J.K. Patra, G. Das, S. Lee, S.-S. Kang, H.-S. Shin, Selected commercial plants: A review of extraction and isolation of bioactive compounds and their pharmacological market value, Trends in Food Science & Technology, 82 (2018) 89-109 https://doi.org/10.1016/j.tifs.2018.10.001.

[8] G. Brusotti, I. Cesari, A. Dentamaro, G. Caccialanza, G. Massolini, Isolation and characterization of bioactive compounds from plant resources: The role of analysis in the ethnopharmacological approach, Journal of Pharmaceutical and Biomedical Analysis, 87 (2014) 218-228 https://doi.org/10.1016/j.jpba.2013.03.007.

[9] S. Sasidharan, Y. Chen, D. Saravanan, K.M. Sundram, L. Yoga Latha, Extraction, isolation and characterization of bioactive compounds from plants' extracts, African Journal of Traditional, Complementary, and Alternative Medicines, 8 (2011) 1-10 https://doi.org/10.4314/ajtcam.v8i1.6048.

[10] C.M. Ajila, K.A. Naidu, S.G. Bhat, U.J.S.P. Rao, Bioactive compounds and antioxidant potential of mango peel extract, Food Chemistry, 105 (2007) 982-988 https://doi.org/10.1016/j.foodchem.2007.04.052.

[11] F. Chemat, M. Abert-Vian, A.S. Fabiano-Tixier, J. Strube, L. Uhlenbrock, V. Gunjevic, G. Cravotto, Green extraction of natural products. Origins, current status, and future challenges, TrAC Trends in Analytical Chemistry, 118 (2019) 248-263 https://doi.org/10.1016/j.trac.2019.05.037.

[12] G. Cravotto, A. Binello, L. Orio, Green extraction techniques, Agro Food Ind. Hi-tech, 22 (2011) 57-59.

[13] F. Chemat, M.A. Vian, G. Cravotto, Green Extraction of Natural Products: Concept and Principles, International Journal of Molecular Sciences, 13 (2012) 8615-8627 https://doi.org/10.3390/ijms13078615.

[14] M. Cvjetko Bubalo, S. Vidović, I. Radojčić Redovniković, S. Jokić, New perspective in extraction of plant biologically active compounds by green solvents, Food and Bioproducts Processing, 109 (2018) 52-73 https://doi.org/10.1016/j.fbp.2018.03.001.

[15] H.J.B.a.S. Pilz, Extraction of Natural Products from Plants – An Introduction, in: Industrial Scale Natural Products Extraction, 2011, pp. 1-25 ISBN: 9783527635122.

[16] D.E. Wedge, J.C.G. Galindo, F.A. Macías, Fungicidal activity of natural and synthetic sesquiterpene lactone analogs, Phytochemistry, 53 (2000) 747-757 https://doi.org/10.1016/S0031-9422(00)00008-X.

[17] A.K. Picman, Biological activities of sesquiterpene lactones, Biochemical Systematics and Ecology, 14 (1986) 255-281 https://doi.org/10.1016/0305-1978(86)90101-8.

[18] P.A.B. Ramos, Â.R. Guerra, O. Guerreiro, S.A.O. Santos, H. Oliveira, C.S.R. Freire, A.J.D. Silvestre, M.F. Duarte, Antiproliferative Effects of Cynara cardunculus L. var. altilis (DC) Lipophilic Extracts, International Journal of Molecular Sciences, 18 (2016) 63 https://doi.org/10.3390/ijms18010063.

[19] A.E. Abe, C.E. de Oliveira, T.M. Dalboni, D.A. Chagas-Paula, B.A. Rocha, R.B. de Oliveira, T.H. Gasparoto, F.B. Da Costa, A.P. Campanelli, Anti-inflammatory sesquiterpene lactones from Tithonia diversifolia trigger different effects on human neutrophils, Revista Brasileira de Farmacognosia, 25 (2015) 111-116 https://doi.org/10.1016/j.bjp.2015.01.005.

[20] S.M. Adekenov, Study of antiopisthorchiasis activity of sesquiterpene lactones and their derivatives, Fitoterapia, 133 (2019) 200-211 https://doi.org/10.1016/j.fitote.2018.12.017.

[21] M. Aghaei, M. Ghanadian, S.E. Sajjadi, R. Saghafian, M. Keyvanloo shahrestanaki, Pimpinelol, a novel atypical Sesquiterpene lactone from Pimpinella haussknechtii fruits with evaluation of endoplasmic reticulum stress in breast cancer cells, Fitoterapia, 129 (2018) 198-202 https://doi.org/10.1016/j.fitote.2018.06.024.

[22] A. Bachelier, R. Mayer, C.D. Klein, Sesquiterpene lactones are potent and irreversible inhibitors of the antibacterial target enzyme MurA, Bioorganic & Medicinal Chemistry Letters, 16 (2006) 5605-5609 https://doi.org/10.1016/j.bmcl.2006.08.021.

[23] V.-B. Bui, S.-T. Liu, J.-J. Zhu, J. Xiong, Y. Zhao, G.-X. Yang, G. Xia, J.-F. Hu, Sesquiterpene lactones from the aerial parts of Xanthium sibiricum and their cytotoxic effects on human cancer cell lines, Phytochemistry Letters, 5 (2012) 685-689 https://doi.org/10.1016/j.phytol.2012.07.008.

[24] L.A. Caldas, M.L. Yoshinaga, M.J.P. Ferreira, J.H.G. Lago, A.B. de Souza, M.D. Laurenti, L.F.D. Passero, P. Sartorelli, Antileishmanial activity and ultrastructural changes of sesquiterpene lactones isolated from Calea pinnatifida (Asteraceae), Bioorganic Chemistry, 83 (2019) 348-353 https://doi.org/10.1016/j.bioorg.2018.10.059.

[25] J.Y. Cho, A.R. Kim, H.-G. Joo, B.-h. Kim, M.H. Rhee, E.S. Yoo, D.R. Katz, B.M. Chain, J.H. Jung, Cynaropicrin, a sesquiterpene lactone, as a new strong regulator of CD29 and CD98 functions, Biochemical and Biophysical Research Communications, 313 (2004) 954-961 https://doi.org/10.1016/j.bbrc.2003.12.026.

[26] J.Y. Cho, A.R. Kim, J.H. Jung, T. Chun, M.H. Rhee, E.S. Yoo, Cytotoxic and pro-apoptotic activities of cynaropicrin, a sesquiterpene lactone, on the viability of leukocyte cancer cell lines, European Journal of Pharmacology, 492 (2004) 85-94 https://doi.org/10.1016/j.ejphar.2004.03.027.

[27] J. Fernández, M.D. Curt, P.L. Aguado, Industrial applications of Cynara cardunculus L. for energy and other uses, Industrial Crops and Products, 24 (2006) 222-229 https://doi.org/10.1016/j.indcrop.2006.06.010.

[28] J. Gominho, A. Lourenço, P. Palma, M.E. Lourenço, M.D. Curt, J. Fernández, H. Pereira, Large scale cultivation of Cynara cardunculus L. for biomass production—A case study, Industrial Crops and Products, 33 (2011) 1-6 https://doi.org/10.1016/j.indcrop.2010.09.011.

[29] P.A.B. Ramos, Â.R. Guerra, O. Guerreiro, C.S.R. Freire, A.M.S. Silva, M.F. Duarte, A.J.D. Silvestre, Lipophilic Extracts of Cynara cardunculus L. var. altilis (DC): A Source of Valuable Bioactive Terpenic Compounds, Journal of Agricultural and Food Chemistry, 61 (2013) 8420-8429 https://doi.org/10.1021/jf402253a.

[30] H. Shimoda, K. Ninomiya, N. Nishida, T. Yoshino, T. Morikawa, H. Matsuda, M. Yoshikawa, Anti-Hyperlipidemic sesquiterpenes and new sesquiterpene glycosides from the leaves of artichoke (Cynara scolymus L.): structure requirement and mode of action, Bioorganic & Medicinal Chemistry Letters, 13 (2003) 223-228 https://doi.org/10.1016/S0960-894X(02)00889-2.

[31] T. Usuki, M. Sato, S. Hara, Y. Yoshimoto, R. Kondo, S. Zimmermann, M. Kaiser, R. Brun, M. Hamburger, M. Adams, Antitrypanosomal structure–activity-relationship study of synthetic cynaropicrin derivatives, Bioorganic & Medicinal Chemistry Letters, 24 (2014) 794-798 https://doi.org/10.1016/j.bmcl.2013.12.099.

[32] J. Cis, G. Nowak, W. Kisiel, Antifeedant properties and chemotaxonomic implications of sesquiterpene lactones and syringin from Rhaponticum pulchrum, Biochemical Systematics and Ecology, 34 (2006) 862-867 https://doi.org/10.1016/j.bse.2006.05.019.

[33] F. Emendörfer, F. Emendörfer, F. Bellato, V.F. Noldin, V. Cechinel-Filho, R.A. Yunes, F.D. Monache, A.M. Cardozo, Antispasmodic Activity of Fractions and Cynaropicrin from Cynara scolymus on Guinea-Pig Ileum, Biological and Pharmaceutical Bulletin, 28 (2005) 902-904 https://doi.org/10.1248/bpb.28.902.

[34] Y.T. Tanaka, K. Tanaka, H. Kojima, T. Hamada, T. Masutani, M. Tsuboi, Y. Akao, Cynaropicrin from Cynara scolymus L. suppresses photoaging of skin by inhibiting the transcription activity of nuclear factor-kappa B, Bioorganic & Medicinal Chemistry Letters, 23 (2013) 518-523 https://doi.org/10.1016/j.bmcl.2012.11.034.

[35] J.Y. Cho, K.U. Baik, J.H. Jung, M.H. Park, In vitro anti-inflammatory effects of cynaropicrin, a sesquiterpene lactone, from Saussurea lappa, European Journal of Pharmacology, 398 (2000) 399-407 https://doi.org/10.1016/S0014-2999(00)00337-X.

[36] C. Formisano, C. Sirignano, D. Rigano, G. Chianese, G. Zengin, E.-J. Seo, T. Efferth, O. Taglialatela-Scafati, Antiproliferative activity against leukemia cells of sesquiterpene lactones from the Turkish endemic plant Centaurea drabifolia subsp. detonsa, Fitoterapia, 120 (2017) 98-102 https://doi.org/10.1016/j.fitote.2017.05.016.

[37] A.M. Ferro, P. Ramos, Â. Guerra, P. Parreira, T. Brás, O. Guerreiro, E. Jerónimo, C. Capel, J. Capel, F.J. Yuste-Lisbona, M.F. Duarte, R. Lozano, M.M. Oliveira, S. Gonçalves, Haplotype analysis of the germacrene A synthase gene and association with cynaropicrin content and biological activities in Cynara cardunculus, Molecular Genetics and Genomics, 293 (2018) 417-433 https://doi.org/10.1007/s00438-017-1388-z

[38] O.M. Andersen, K.R. Markham, Flavonoids: chemistry, biochemistry and applications, CRC press, 2005 ISBN: 142003944X.

[39] M.E. Cuvelier, H. Richard, C. Berset, Antioxidative activity and phenolic composition of pilot-plant and commercial extracts of sage and rosemary, Journal of the American Oil Chemists' Society, 73 (1996) 645-652 https://doi.org/10.1007/BF02518121.

[40] M. Suhaj, Spice antioxidants isolation and their antiradical activity: a review, Journal of Food Composition and Analysis, 19 (2006) 531-537 https://doi.org/10.1016/j.jfca.2004.11.005.

[41] A.S. Adekenova, P.Y. Sakenova, S.A. Ivasenko, I.A. Khabarov, S.M. Adekenov, A. Berthod, Gram-Scale Purification of Two Sesquiterpene Lactones from Chartolepsis Intermedia Boiss, Chromatographia, 79 (2016) 37-43 https://doi.org/10.1007/s10337-015-3000-1.

[42] V. Milutinović, M. Niketić, L. Ušjak, D. Nikolić, A. Krunić, C. Zidorn, S. Petrović, Methanol Extracts of 28 Hieracium Species from the Balkan Peninsula – Comparative LC–MS Analysis, Chemosystematic Evaluation of their Flavonoid and Phenolic Acid Profiles and Antioxidant Potentials, Phytochemical Analysis, 29 (2018) 30-47 https://doi.org/10.1002/pca.2712.

[43] J. Nawrot, J. Budzianowski, G. Nowak, Phytochemical profiles of the leaves of Stizolophus balsamita and Psephellus sibiricus and their chemotaxonomic implications, Phytochemistry, 159 (2019) 172-178 https://doi.org/10.1016/j.phytochem.2018.12.022.

[44] E. Conde, A. Moure, H. Domínguez, J.C. Parajó, 18 - Extraction of natural antioxidants from plant foods, in: S.S.H. Rizvi (Ed.) Separation, Extraction and Concentration Processes in the Food, Beverage and Nutraceutical Industries, Woodhead Publishing, 2013, pp. 506-594 ISBN: 978-1-84569-645-0.

[45] J. Li, H.A. Chase, Applications of membrane techniques for purification of natural products, Biotechnology Letters, 32 (2010) 601-608 https://doi.org/10.1007/s10529-009-0199-7.

[46] O. Akin, F. Temelli, S. Köseoğlu, Membrane Applications in Functional Foods and Nutraceuticals, Critical Reviews in Food Science and Nutrition, 52 (2012) 347-371 https://doi.org/10.1080/10408398.2010.500240.

[47] E. Drioli, M. Romano, Progress and New Perspectives on Integrated Membrane Operations for Sustainable Industrial Growth, Industrial & Engineering Chemistry Research, 40 (2001) 1277-1300 https://doi.org/10.1021/ie0006209.

[48] E.A. Baptista, P.C.R. Pinto, I.F. Mota, J.M. Loureiro, A.E. Rodrigues, Ultrafiltration of ethanol/water extract of Eucalyptus globulus bark: Resistance and cake build up analysis, Separation and Purification Technology, 144 (2015) 256-266 https://doi.org/10.1016/j.seppur.2015.02.036.

[49] M.T.C. Machado, S. Trevisan, J.D.R. Pimentel-Souza, G.M. Pastore, M.D. Hubinger, Clarification and concentration of oligosaccharides from artichoke extract by a sequential process with microfiltration and nanofiltration membranes, Journal of Food Engineering, 180 (2016) 120-128 https://doi.org/10.1016/j.jfoodeng.2016.02.018.

[50] A.R.S. Teixeira, J.L.C. Santos, J.G. Crespo, Solvent resistant diananofiltration for production of steryl esters enriched extracts, Separation and Purification Technology, 135 (2014) 243-251 https://doi.org/10.1016/j.seppur.2014.08.007.

[51] B. Tylkowski, I. Tsibranska, R. Kochanov, G. Peev, M. Giamberini, Concentration of biologically active compounds extracted from Sideritis ssp. L. by nanofiltration, Food and Bioproducts Processing, 89 (2011) 307-314 https://doi.org/10.1016/j.fbp.2010.11.003.

[52] J.C.-T. Lin, A.G. Livingston, Nanofiltration membrane cascade for continuous solvent exchange, Chemical Engineering Science, 62 (2007) 2728-2736 https://doi.org/10.1016/j.ces.2006.08.004.

[53] I. Sereewatthanawut, F.W. Lim, Y.S. Bhole, D. Ormerod, A. Horvath, A.T. Boam, A.G. Livingston, Demonstration of Molecular Purification in Polar Aprotic Solvents by Organic Solvent Nanofiltration, Organic Process Research & Development, 14 (2010) 600-611 https://doi.org/10.1021/op100028p.

[54] A. Tsoukala, L. Peeva, A.G. Livingston, H.-R. Bjørsvik, Separation of Reaction Product and Palladium Catalyst after a Heck Coupling Reaction by means of Organic Solvent Nanofiltration, ChemSusChem, 5 (2012) 188-193 https://doi.org/10.1002/cssc.201100355.

[55] E.M. Rundquist, C.J. Pink, A.G. Livingston, Organic solvent nanofiltration: a potential alternative to distillation for solvent recovery from crystallisation mother liquors, Green Chemistry, 14 (2012) 2197-2205 https://doi.org/10.1039/c2gc35216h.

[56] D. Peshev, L.G. Peeva, G. Peev, I.I.R. Baptista, A.T. Boam, Application of organic solvent nanofiltration for concentration of antioxidant extracts of rosemary (Rosmarinus officiallis L.), Chemical Engineering Research and Design, 89 (2011) 318-327 https://doi.org/10.1016/j.cherd.2010.07.002.

[57] F. Abdallah, L. Mijouin, C. Pichon, Skin Immune Landscape: Inside and Outside the Organism, Mediators of inflammation, 2017 (2017) 5095293-5095293 https://doi.org/10.1155/2017/5095293.

[58] G. Schoukens, 5 - Bioactive dressings to promote wound healing, in: S. Rajendran (Ed.) Advanced Textiles for Wound Care, Woodhead Publishing, 2009, pp. 114-152 ISBN: 978-1-84569-271-1.

[59] R.A.F. Clark, Overview and General Considerations of Wound Repair, in: R.A.F. Clark, P.M. Henson (Eds.) The Molecular and Cellular Biology of Wound Repair, Springer US, Boston, MA, 1988, pp. 3-33 ISBN: 978-1-4615-1795-5.

[60] R.L. Harries, D.C. Bosanquet, K.G. Harding, Wound bed preparation: TIME for an update, International Wound Journal, 13 (2016) 8-14 https://doi.org/10.1111/iwj.12662.

[61] S. Saghazadeh, C. Rinoldi, M. Schot, S.S. Kashaf, F. Sharifi, E. Jalilian, K. Nuutila, G. Giatsidis, P. Mostafalu, H. Derakhshandeh, K. Yue, W. Swieszkowski, A. Memic, A. Tamayol, A. Khademhosseini, Drug delivery systems and materials for wound healing applications, Advanced Drug Delivery Reviews, 127 (2018) 138-166 https://doi.org/10.1016/j.addr.2018.04.008.

[62] N. Mayet, Y.E. Choonara, P. Kumar, L.K. Tomar, C. Tyagi, L.C. Du Toit, V. Pillay, A Comprehensive Review of Advanced Biopolymeric Wound Healing Systems, Journal of Pharmaceutical Sciences, 103 (2014) 2211-2230 https://doi.org/10.1002/jps.24068.

[63] R. Zhao, H. Liang, E. Clarke, C. Jackson, M. Xue, Inflammation in Chronic Wounds, International Journal of Molecular Sciences, 17 (2016) 2085 https://doi.org/10.3390/ijms17122085.

[64] J.S. Boateng, K.H. Matthews, H.N.E. Stevens, G.M. Eccleston, Wound Healing Dressings and Drug Delivery Systems: A Review, Journal of Pharmaceutical Sciences, 97 (2008) 2892-2923 https://doi.org/10.1002/jps.21210.

[65] W. Paul, C.P. Sharma, Chitosan and alginate wound dressings: a short review, Trends Biomaterial Artificial Organs, 18 (2004) 18-23.

[66] A.S. Halim, F.M. Nor, A.Z. Mat Saad, N.A. Mohd Nasir, B. Norsa'adah, Z. Ujang, Efficacy of chitosan derivative films versus hydrocolloid dressing on superficial wounds, Journal of Taibah University Medical Sciences, 13 (2018) 512-520 https://doi.org/10.1016/j.jtumed.2018.10.004.

[67] Y. Fujimoto, N. Shimooka, Y.-i. Ohnishi, T. Yoshimine, Clinical evaluation of hydrocolloid dressings for neurosurgical wounds, Surgical Neurology, 70 (2008) 217-220 https://doi.org/10.1016/j.surneu.2007.06.091.

[68] K. Varaprasad, T. Jayaramudu, V. Kanikireddy, C. Toro, E.R. Sadiku, Alginate-based composite materials for wound dressing application:A mini review, Carbohydrate Polymers, 236 (2020) 116025 https://doi.org/10.1016/j.carbpol.2020.116025.

[69] A. Moeini, P. Pedram, P. Makvandi, M. Malinconico, G. Gomez d'Ayala, Wound healing and antimicrobial effect of active secondary metabolites in chitosan-based wound dressings: A review, Carbohydrate Polymers, 233 (2020) 115839 https://doi.org/10.1016/j.carbpol.2020.115839.

[70] Y. Wang, C. Fu, Z. Wu, L. Chen, X. Chen, Y. Wei, P. Zhang, A chitin film containing basic fibroblast growth factor with a chitin-binding domain as wound dressings, Carbohydrate Polymers, 174 (2017) 723-730 https://doi.org/10.1016/j.carbpol.2017.05.087.

[71] E. Duncan, 7 - Regulation of biotextiles for medical use, in: M.W. King, B.S. Gupta, R. Guidoin (Eds.) Biotextiles as Medical Implants, Woodhead Publishing, 2013, pp. 169-181 ISBN: 978-1-84569-439-5.

[72] M. Aider, Chitosan application for active bio-based films production and potential in the food industry: Review, LWT - Food Science and Technology, 43 (2010) 837-842 https://doi.org/10.1016/j.lwt.2010.01.021.

[73] A.R.V. Ferreira, N.M. Bandarra, M. Moldão-Martins, I.M. Coelhoso, V.D. Alves, FucoPol and chitosan bilayer films for walnut kernels and oil preservation, LWT - Food Science and Technology, 91 (2018) 34-39 https://doi.org/10.1016/j.lwt.2018.01.020.

[74] T. de Moraes Crizel, A. de Oliveira Rios, V. D. Alves, N. Bandarra, M. Moldão-Martins, S. Hickmann Flôres, Active food packaging prepared with chitosan and olive pomace, Food Hydrocolloids, 74 (2018) 139-150 https://doi.org/10.1016/j.foodhyd.2017.08.007.

[75] A.R.V. Ferreira, V.D. Alves, I.M. Coelhoso, Polysaccharide-Based Membranes in Food Packaging Applications, Membranes, 6 (2016) 22 https://doi.org/10.3390/membranes6020022.

[76] R.A.A. Muzzarelli, Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone, Carbohydrate Polymers, 76 (2009) 167-182 https://doi.org/10.1016/j.carbpol.2008.11.002.

[77] A. Ali, S. Ahmed, A review on chitosan and its nanocomposites in drug delivery, International Journal of Biological Macromolecules, 109 (2018) 273-286 https://doi.org/10.1016/j.ijbiomac.2017.12.078.

[78] J. Li, C. Cai, J. Li, J. Li, J. Li, T. Sun, L. Wang, H. Wu, G. Yu, Chitosan-Based Nanomaterials for Drug Delivery, Molecules (Basel, Switzerland), 23 (2018) 2661 https://doi.org/10.3390/molecules23102661.

[79] A. Bernkop-Schnürch, S. Dünnhaupt, Chitosan-based drug delivery systems, European Journal of Pharmaceutics and Biopharmaceutics, 81 (2012) 463-469 https://doi.org/10.1016/j.ejpb.2012.04.007.

[80] M.A. Bezerra, R.E. Santelli, E.P. Oliveira, L.S. Villar, L.A. Escaleira, Response surface methodology (RSM) as a tool for optimization in analytical chemistry, Talanta, 76 (2008) 965-977 https://doi.org/10.1016/j.talanta.2008.05.019.

[81] Z.-Q. Lin, T. Kondo, Y. Ishida, T. Takayasu, N. Mukaida, Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice, Journal of Leukocyte Biology, 73 (2003) 713-721 https://doi.org/10.1189/jlb.0802397.

[82] E. Rodriguez, G.H.N. Towers, J.C. Mitchell, Biological activities of sesquiterpene lactones, Phytochemistry, 15 (1976) 1573-1580 https://doi.org/10.1016/S0031-9422(00)97430-2.

[83] O. Spring, J. Kupka, B. Maier, A. Hager, Biological Activities of Sesquiterpene Lactones from Helianthus annuus: Antimicrobial and Cytotoxic Properties; Influence on DNA, RNA, and Protein Synthesis, in: Zeitschrift für Naturforschung C, 1982, pp. 1087, https://doi.org/10.1515/znc-1982-11-1207.

[84] M. Chadwick, H. Trewin, F. Gawthrop, C. Wagstaff, Sesquiterpenoids lactones: benefits to plants and people, International Journal of Molecular Sciences, 14 (2013) 12780-12805 https://doi.org/10.3390/ijms140612780.

[85] A. Beharav, A. Stojakowska, R. Ben-David, J. Malarz, K. Michalska, W. Kisiel, Variation of sesquiterpene lactone contents in Lactuca georgica natural populations from Armenia, Genetic Resources and Crop Evolution, 62 (2015) 431-441 https://doi.org/10.1007/s10722-014-0171-9.

[86] J. Fu, J. Yu, J. Chen, H. Xu, Y. Luo, H. Lu, In vitro inhibitory properties of sesquiterpenes from Chloranthus serratus on cell motility via down-regulation of LIMK1 activation in human breast cancer, Phytomedicine, 49 (2018) 23-31 https://doi.org/10.1016/j.phymed.2018.06.009.

[87] L.-S. Gan, Y.-L. Zheng, J.-X. Mo, X. Liu, X.-H. Li, C.-X. Zhou, Sesquiterpene Lactones from the Root Tubers of Lindera aggregata, Journal of Natural Products, 72 (2009) 1497-1501 https://doi.org/10.1021/np900354q.

[88] H.-L. Huang, Y.-J. Xu, H.-L. Liu, X.-Q. Liu, J.-N. Shang, G.-T. Han, M.-J. Yao, C.-S. Yuan, Eremophilane-type sesquiterpene lactones from Ligularia hodgsonii Hook, Phytochemistry, 72 (2011) 514-517 https://doi.org/10.1016/j.phytochem.2011.01.008.

[89] S.-i. Kurimoto, K. Suzuki, M. Okasaka, Y. Kashiwada, O.K. Kodzhimatov, Y. Takaishi, New sesquiterpene lactone glucosides from the roots of Ferula varia, Phytochemistry Letters, 5 (2012) 729-733 https://doi.org/10.1016/j.phytol.2012.08.001.

[90] Q. Liu, J.H. Ahn, S.B. Kim, C. Lee, B.Y. Hwang, M.K. Lee, Sesquiterpene lactones from the roots of Lindera strychnifolia, Phytochemistry, 87 (2013) 112-118 https://doi.org/10.1016/j.phytochem.2012.11.004.

[91] K. Michalska, E. Szneler, W. Kisiel, Sesquiterpene lactones from Lactuca canadensis and their chemotaxonomic significance, Phytochemistry, 90 (2013) 90-94 https://doi.org/10.1016/j.phytochem.2013.02.005.

[92] S. Mohan, D. Gupta, Phytochemical analysis and differential in vitro cytotoxicity assessment of root extracts of Inula racemosa, Biomedicine & Pharmacotherapy, 89 (2017) 781-795 https://doi.org/10.1016/j.biopha.2017.02.053.

[93] K. Polatoğlu, Ö.C. Karakoç, A. Gökçe, N. Gören, Insecticidal activity of Tanacetum chiliophyllum (Fisch. & Mey.) var. monocephalum grierson extracts and a new sesquiterpene lactone, Phytochemistry Letters, 4 (2011) 432-435 https://doi.org/10.1016/j.phytol.2011.06.005.

[94] K. Sowndhararajan, H. Cho, B. Yu, J. Song, S. Kim, Effect of inhalation of essential oil from Inula helenium L. root on electroencephalographic (EEG) activity of the human brain, European Journal of Integrative Medicine, 8 (2016) 453-457 https://doi.org/10.1016/j.eujim.2016.01.005.

[95] H. Sumioka, L. Harinantenaina, K. Matsunami, H. Otsuka, M. Kawahata, K. Yamaguchi, Linderolides A–F, eudesmane-type sesquiterpene lactones and linderoline, a germacrane-type sesquiterpene from the roots of Lindera strychnifolia and their inhibitory activity on NO production in RAW 264.7 cells in vitro, Phytochemistry, 72 (2011) 2165-2171 https://doi.org/10.1016/j.phytochem.2011.08.004.

[96] T. Zhang, T. Gong, Y. Yang, R.-Y. Chen, D.-Q. Yu, Two new eudesmanolides from Inula racemosa and their bioactivities, Phytochemistry Letters, 5 (2012) 229-232 https://doi.org/10.1016/j.phytol.2011.12.014.

[97] E.P. Clark, Helenalin. I. Helenalin, the Bitter Sternutative Substance Occurring in Helenium Autumnale, Journal of the American Chemical Society, 58 (1936) 1982-1983 https://doi.org/10.1021/ja01301a046.

[98] Z. Abdolmaleki, H.-A. Arab, S. Amanpour, S. Muhammadnejad, Anti-angiogenic effects of ethanolic extract of Artemisia sieberi compared to its active substance, artemisinin, Revista Brasileira de Farmacognosia, 26 (2016) 326-333 https://doi.org/10.1016/j.bjp.2015.11.008.

[99] N. Amat, H. Upur, B. Blažeković, In vivo hepatoprotective activity of the aqueous extract of Artemisia absinthium L. against chemically and immunologically induced liver injuries in mice, Journal of Ethnopharmacology, 131 (2010) 478-484 https://doi.org/10.1016/j.jep.2010.07.023.

[100] B. Bach, M. Cleroux, M. Saillen, P. Schönenberger, S. Burgos, J. Ducruet, A. Vallat, A new chemical tool for absinthe producers, quantification of α/β-thujone and the bitter components in Artemisia absinthium, Food Chemistry, 213 (2016) 813-817 https://doi.org/10.1016/j.foodchem.2016.06.045.

[101] L. Baldino, E. Reverchon, G. Della Porta, An optimized process for SC-CO2 extraction of antimalarial compounds from Artemisia annua L, The Journal of Supercritical Fluids, 128 (2017) 89-93 https://doi.org/10.1016/j.supflu.2017.05.018.

[102] R. Briars, L. Paniwnyk, Effect of ultrasound on the extraction of artemisinin from Artemisia annua, Industrial Crops and Products, 42 (2013) 595-600 https://doi.org/10.1016/j.indcrop.2012.06.043.

[103] S. Chemat, A. Aissa, A. Boumechhour, O. Arous, H. Ait-Amar, Extraction mechanism of ultrasound assisted extraction and its effect on higher yielding and purity of artemisinin crystals from Artemisia annua L. leaves, Ultrasonics Sonochemistry, 34 (2017) 310-316 https://doi.org/10.1016/j.ultsonch.2016.05.046.

[104] J. Chi, B.-c. Li, W.-f. Dai, L. Liu, M. Zhang, Highly oxidized sesquiterpenes from Artemisia austro-yunnanensis, Fitoterapia, 115 (2016) 182-188 https://doi.org/10.1016/j.fitote.2016.10.013.

[105] J.Y. Cho, S.J. Jeong, H. Lee, K.H. Park, D.Y. Hwang, S.Y. Park, Y.G. Lee, J.H. Moon, K.S. Ham, Sesquiterpene lactones and scopoletins from Artemisia scoparia Waldst. & Kit. and their angiotensin I-converting enzyme inhibitory activities, Food Science and Biotechnology, 25 (2016) 1701-1708 https://doi.org/10.1007/s10068-016-0261-x.

[106] J.F.S. Ferreira, V.D. Zheljazkov, J.M. Gonzalez, Artemisinin concentration and antioxidant capacity of Artemisia annua distillation byproduct, Industrial Crops and Products, 41 (2013) 294-298 https://doi.org/10.1016/j.indcrop.2012.05.005.

[107] A. Gonzalez-Coloma, M. Bailen, C.E. Diaz, B.M. Fraga, R. Martínez-Díaz, G.E. Zuñiga, R.A. Contreras, R. Cabrera, J. Burillo, Major components of Spanish cultivated Artemisia absinthium populations: Antifeedant, antiparasitic, and antioxidant effects, Industrial Crops and Products, 37 (2012) 401-407 https://doi.org/10.1016/j.indcrop.2011.12.025.

[108] J.-y. Hao, W. Han, S.-d. Huang, B.-y. Xue, X. Deng, Microwave-assisted extraction of artemisinin from Artemisia annua L, Separation and Purification Technology, 28 (2002) 191-196 https://doi.org/10.1016/S1383-5866(02)00043-6.

[109] L. Li, H. Liu, C. Tang, S. Yao, C. Ke, C. Xu, Y. Ye, Cytotoxic sesquiterpene lactones from Artemisia anomala, Phytochemistry Letters, 20 (2017) 177-180 https://doi.org/10.1016/j.phytol.2017.04.038.

[110] M.G. Phan, T.T. Nhan Tran, T.S. Phan, H. Otsuka, K. Matsunami, Two new sesquiterpene lactones and other chemical constituents of Artemisia roxbughiana, Biochemical Systematics and Ecology, 45 (2012) 115-119 https://doi.org/10.1016/j.bse.2012.07.027.

[111] I.D. Sham'yanov, B. Tashkhodzhaev, R.F. Mukhamatkhanova, M.N. Sultankhodzhaev, M.G. Levkovich, N.D. Abdullaev, M.Y. Antipin, Sesquiterpene lactones and new diterpenoid alkaloids from Artemisia korshinskyi, Chemistry of Natural Compounds, 48 (2012) 616-621 https://doi.org/10.1007/s10600-012-0326-x.

[112] M. Shoaib, I. Shah, N. Ali, A. Adhikari, M.N. Tahir, S.W.A. Shah, S. Ishtiaq, J. Khan, S. Khan, M.N. Umer, Sesquiterpene lactone! a promising antioxidant, anticancer and moderate antinociceptive agent from Artemisia macrocephala jacquem, BMC Complementary and Alternative Medicine, 17 (2017) 27-27 https://doi.org/10.1186/s12906-016-1517-y.

[113] E.d.O. Silva, L.L. Borges, E.C.d. Conceição, M.T.F. Bara, Box–Behnken experimental design for extraction of artemisinin from Artemisia annua and validation of the assay method, Revista Brasileira de Farmacognosia, 27 (2017) 519-524 https://doi.org/10.1016/j.bjp.2017.03.002.

[114] H. Yin, A. Kjaer, X.C. Fretté, Y. Du, L.P. Christensen, M. Jensen, K. Grevsen, Chitosan oligosaccharide and salicylic acid up-regulate gene expression differently in relation to the biosynthesis of artemisinin in Artemisia annua L, Process Biochemistry, 47 (2012) 1559-1562 https://doi.org/10.1016/j.procbio.2011.12.020.

[115] E.L.P. de Faria, M.V. Gomes, A.F.M. Cláudio, C.S.R. Freire, A.J.D. Silvestre, M.G. Freire, Extraction and recovery processes for cynaropicrin from Cynara cardunculus L. using aqueous solutions of surface-active ionic liquids, Biophysical Reviews, 10 (2018) 915-925 https://doi.org/10.1007/s12551-017-0387-y.

[116] E.L.P. de Faria, R.S. do Carmo, A.F.M. Cláudio, C.S.R. Freire, M.G. Freire, A.J.D. Silvestre, Deep Eutectic Solvents as Efficient Media for the Extraction and Recovery of Cynaropicrin from Cynara cardunculus L. Leaves, International Journal of Molecular Sciences, 18 (2017) 2276 https://doi.org/10.3390/ijms18112276.

[117] A. Scavo, G. Pandino, C. Restuccia, L. Parafati, G. Cirvilleri, G. Mauromicale, Antimicrobial activity of cultivated cardoon (Cynara cardunculus L. var. altilis DC.) leaf extracts against bacterial species of agricultural and food interest, Industrial Crops and Products, 129 (2019) 206-211 https://doi.org/10.1016/j.indcrop.2018.12.005.

[118] A. Scavo, C. Rial, J.M.G. Molinillo, R.M. Varela, G. Mauromicale, F.A. Macias, The extraction procedure improves the allelopathic activity of cardoon (Cynara cardunculus var. altilis) leaf allelochemicals, Industrial Crops and Products, 128 (2019) 479-487 https://doi.org/10.1016/j.indcrop.2018.11.053.

[119] L.E. De Faria, S.R. Do Carmo, M.A.F. Cláudio, S.C. Freire, G.M. Freire, J.A. Silvestre, Deep Eutectic Solvents as Efficient Media for the Extraction and Recovery of Cynaropicrin from Cynara cardunculus L. Leaves, International Journal of Molecular Sciences, 18 (2017) https://doi.org/10.3390/ijms18112276.

[120] J. Fritsche, C.M. Beindorff, M. Dachtler, H. Zhang, J.G. Lammers, Isolation, characterization and determination of minor artichoke (Cynara scolymus L.) leaf extract compounds, European Food Research and Technology, 215 (2002) 149-157 https://doi.org/10.1007/s00217-002-0507-0.

[121] P. Ramos, A. Guerra, O. Guerreiro, S. Santos, H. Oliveira, C. Freire, S. Rocha, A. Silvestre, M.F. Duarte, Antitumoral and antioxidant activities of lipophilic and phenolic extracts from Cynara cardunculus L. var. altilis (DC), Planta Med, 80 (2014) P1L16 https://doi.org/10.1055/s-0034-1394674.

[122] T. Brás, L.A. Neves, J.G. Crespo, M.F. Duarte, Effect of extraction methodologies and solvent selection upon cynaropicrin extraction from Cynara cardunculus leaves, Separation and Purification Technology, 236 (2020) 116283 https://doi.org/10.1016/j.seppur.2019.116283.

[123] J.T. Fischedick, M. Pesic, A. Podolski-Renic, J. Bankovic, R.C.H. de Vos, M. Perić, S. Todorović, N. Tanic, Cytotoxic activity of sesquiterpene lactones from Inula britannica on human cancer cell lines, Phytochemistry Letters, 6 (2013) 246-252 https://doi.org/10.1016/j.phytol.2013.02.006.

[124] E. Garayev, G. Herbette, C. Di Giorgio, P. Chiffolleau, D. Roux, H. Sallanon, E. Ollivier, R. Elias, B. Baghdikian, New sesquiterpene acid and inositol derivatives from Inula montana L, Fitoterapia, 120 (2017) 79-84 https://doi.org/10.1016/j.fitote.2017.05.011.

[125] N. Kheyar-Kraouche, A.B. da Silva, A.T. Serra, F. Bedjou, M.R. Bronze, Characterization by liquid chromatography–mass spectrometry and antioxidant activity of an ethanolic extract of Inula viscosa leaves, Journal of Pharmaceutical and Biomedical Analysis, 156 (2018) 297-306 https://doi.org/10.1016/j.jpba.2018.04.047.

[126] A. Stojakowska, J. Malarz, W. Kisiel, Quantitative analysis of sesquiterpene lactones and thymol derivatives in extracts from Telekia speciosa, Phytochemistry Letters, 11 (2015) 378-383 https://doi.org/10.1016/j.phytol.2014.10.025.

[127] A. Trendafilova, V. Ivanova, M. Todorova, I. Aneva, New sesquiterpene lactones from Inula oculus-christi L, Phytochemistry Letters, 21 (2017) 221-225 https://doi.org/10.1016/j.phytol.2017.07.008.

[128] F.-Y. Wang, X.-Q. Li, Q. Sun, S. Yao, C.-Q. Ke, C.-P. Tang, H.-C. Liu, M.-Y. Geng, Y. Ye, Sesquiterpene lactones from Inula cappa, Phytochemistry Letters, 5 (2012) 639-642 https://doi.org/10.1016/j.phytol.2012.06.012.

[129] X. Wang, S.A. Tang, R. Wang, Y. Qiu, M. Jin, D. Kong, Inhibitory Effects of JEUD-38, a New Sesquiterpene Lactone from Inula japonica Thunb, on LPS-Induced iNOS Expression in RAW264.7 Cells, Inflammation, 38 (2015) 941-948 https://doi.org/10.1007/s10753-014-0056-2.

[130] J.-W. Wu, C.-P. Tang, Y.-Y. Cai, C.-Q. Ke, L.-G. Lin, S. Yao, Y. Ye, Cytotoxic germacrane-type sesquiterpene lactones from the whole plant of Inula cappa, Chinese Chemical Letters, 28 (2017) 927-930 https://doi.org/10.1016/j.cclet.2016.11.011.

[131] G.G.L. Yue, B.C.L. Chan, H.-F. Kwok, Y.-L. Wong, H.-W. Leung, C.-J. Ji, K.-P. Fung, P.-C. Leung, N.-H. Tan, C.B.S. Lau, Anti-angiogenesis and immunomodulatory activities of an anti-tumor sesquiterpene bigelovin isolated from Inula helianthus-aquatica, European Journal of Medicinal Chemistry, 59 (2013) 243-252 https://doi.org/10.1016/j.ejmech.2012.11.029.

[132] G. Negrín, S. Rubio, M.T. Marrero, J. Quintana, J.L. Eiroa, J. Triana, F. Estévez, The eudesmanolide tanapsin from Tanacetum oshanahanii and its acetate induce cell death in human tumor cells through a mechanism dependent on reactive oxygen species, Phytomedicine, 22 (2015) 385-393 https://doi.org/10.1016/j.phymed.2015.01.008.

[133] M.F. Rabito, E.A. Britta, B.L. Pelegrini, D.B. Scariot, M.B. Almeida, S.L. Nixdorf, C.V. Nakamura, I.C.P. Ferreira, In vitro and in vivo antileishmania activity of sesquiterpene lactone-rich dichloromethane fraction obtained from Tanacetum parthenium (L.) Schultz-Bip, Experimental Parasitology, 143 (2014) 18-23 https://doi.org/10.1016/j.exppara.2014.04.014.

[134] J. Triana, J.L. Eiroa, M. Morales, F.J. Pérez, I. Brouard, M.T. Marrero, S. Estévez, J. Quintana, F. Estévez, Q.A. Castillo, F. León, A chemotaxonomic study of endemic species of genus Tanacetum from the Canary Islands, Phytochemistry, 92 (2013) 87-104 https://doi.org/10.1016/j.phytochem.2013.04.015.

[135] K. Végh, Á. Alberti, E. Riethmüller, A. Tóth, S. Béni, Á. Kéry, Supercritical fluid extraction and convergence chromatographic determination of parthenolide in Tanacetum parthenium L.: Experimental design, modeling and optimization, The Journal of Supercritical Fluids, 95 (2014) 84-91 https://doi.org/10.1016/j.supflu.2014.07.029.

[136] A.B. Aliyu, B. Moodley, H. Chenia, N.A. Koorbanally, Sesquiterpene lactones from the aerial parts of Vernonia blumeoides growing in Nigeria, Phytochemistry, 111 (2015) 163-168 https://doi.org/10.1016/j.phytochem.2014.11.010.

[137] H. Buskuhl, F.L. de Oliveira, L.Z. Blind, R.A. de Freitas, A. Barison, F.R. Campos, Y.E. Corilo, M.N. Eberlin, G.F. Caramori, M.W. Biavatti, Sesquiterpene lactones from Vernonia scorpioides and their in vitro cytotoxicity, Phytochemistry, 71 (2010) 1539-1544 https://doi.org/10.1016/j.phytochem.2010.06.007.

[138] P.W.C. Green, S.R. Belmain, P.A. Ndakidemi, I.W. Farrell, P.C. Stevenson, Insecticidal activity of Tithonia diversifolia and Vernonia amygdalina, Industrial Crops and Products, 110 (2017) 15-21 https://doi.org/10.1016/j.indcrop.2017.08.021.

[139] M.E. Poleti Martucci, L. Gobbo-Neto, Differential secondary metabolite accumulation and performance of Chlosyne lacinia fed with Tithonia diversifolia or Vernonia polyanthes, Biochemical Systematics and Ecology, 68 (2016) 156-162 https://doi.org/10.1016/j.bse.2016.07.009.

[140] A. Prasopthum, P. Pouyfung, S. Sarapusit, E. Srisook, P. Rongnoparut, Inhibition effects of Vernonia cinerea active compounds against cytochrome P450 2A6 and human monoamine oxidases, possible targets for reduction of tobacco dependence, Drug Metabolism and Pharmacokinetics, 30 (2015) 174-181 https://doi.org/10.1016/j.dmpk.2014.12.005.

[141] N.J. Toyang, R. Verpoorte, A review of the medicinal potentials of plants of the genus Vernonia (Asteraceae), Journal of Ethnopharmacology, 146 (2013) 681-723 https://doi.org/10.1016/j.jep.2013.01.040.

[142] U.J. Youn, G. Miklossy, X. Chai, S. Wongwiwatthananukit, O. Toyama, T. Songsak, J. Turkson, L.C. Chang, Bioactive sesquiterpene lactones and other compounds isolated from Vernonia cinerea, Fitoterapia, 93 (2014) 194-200 https://doi.org/10.1016/j.fitote.2013.12.013.

[143] R. Hendra, S. Ahmad, A. Sukari, M.Y. Shukor, E. Oskoueian, Flavonoid analyses and antimicrobial activity of various parts of Phaleria macrocarpa (Scheff.) Boerl fruit, International Journal of Molecular Sciences, 12 (2011) 3422-3431 https://doi.org/10.3390/ijms12063422.

[144] B. Dreisewerd, J. Merz, G. Schembecker, Determining the solute–solid interactions in phytoextraction, Chemical Engineering Science, 134 (2015) 287-296 https://doi.org/10.1016/j.ces.2015.04.025.

[145] P.A.B. Ramos, Â.R. Guerra, O. Guerreiro, S.A.O. Santos, H. Oliveira, C.S.R. Freire, A.J.D. Silvestre, M.F. Duarte, Antiproliferative Effects of Cynara cardunculus L. var. altilis (DC) Lipophilic Extracts, International Journal of Molecular Sciences, 18 (2017) 63 http://doi.org/10.3390/ijms18010063.

[146] K.H. Teoh, D.R. Polichuk, D.W. Reed, G. Nowak, P.S. Covello, Artemisia annua L. (Asteraceae) trichome-specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin, FEBS Letters, 580 (2006) 1411-1416 https://doi.org/10.1016/j.febslet.2006.01.065.

[147] H.J. Woerdenbag, C.B. Lugt, N. Pras, Artemisia annua L.: a source of novel antimalarial drugs, Pharmaceutisch Weekblad, 12 (1990) 169-181 https://doi.org/10.1007/bf01980041.

[148] H.J. Woerdenbag, N. Pras, W. van Uden, T.E. Wallaart, A.C. Beekman, C.B. Lugt, Progress in the research of artemisinin-related antimalarials: An update, Pharmacy World and Science, 16 (1994) 169-180 https://doi.org/10.1007/bf01872865.

[149] S.K.S. Amoah, E. Kouloura, L.M. Dutra, A. Barison, L.M. Wildner, M.L. Bazzo, M. Halabalaki, L.A. Skaltsounis, M.W. Biavatti, Phytochemical analysis of the hot tea infusion of Hedyosmum brasiliense, Phytochemistry Letters, 13 (2015) 267-274 https://doi.org/10.1016/j.phytol.2015.07.013.

[150] S.K.S. Amoah, F.L. de Oliveira, A.C.H. da Cruz, N.M. de Souza, F.R. Campos, A. Barison, M.W. Biavatti, Sesquiterpene lactones from the leaves of Hedyosmum brasiliense (Chloranthaceae), Phytochemistry, 87 (2013) 126-132 https://doi.org/10.1016/j.phytochem.2012.11.018.

[151] A. Leitolis, S.K.S. Amoah, M.W. Biavatti, J.E. da Silva-Santos, Sesquiterpene lactones from Hedyosmum brasiliense induce in vitro relaxation of rat aorta and corpus cavernosum, Revista Brasileira de Farmacognosia, 26 (2016) 363-368 https://doi.org/10.1016/j.bjp.2016.01.005.

[152] S.B. Genta, W.M. Cabrera, M.I. Mercado, A. Grau, C.A. Catalán, S.S. Sánchez, Hypoglycemic activity of leaf organic extracts from Smallanthus sonchifolius: Constituents of the most active fractions, Chemico-Biological Interactions, 185 (2010) 143-152 https://doi.org/10.1016/j.cbi.2010.03.004.

[153] E.F. de Andrade, D. Carpiné, J.L.A. Dagostin, A. Barison, A.L. Rüdiger, G.I.B. de Muñiz, M.L. Masson, Identification and antimicrobial activity of the sesquiterpene lactone mixture extracted from Smallanthus sonchifolius dried leaves, European Food Research and Technology, 243 (2017) 2155-2161 https://doi.org/10.1007/s00217-017-2918-y.

[154] M.S. Easmin, M.Z.I. Sarker, S. Ferdosh, S.H. Shamsudin, K.B. Yunus, M.S. Uddin, M.M.R. Sarker, M.J.H. Akanda, M.S. Hossain, H.A. Khalil, Bioactive compounds and advanced processing technology: Phaleria macrocarpa (sheff.) Boerl, a review, Journal of Chemical Technology & Biotechnology, 90 (2015) 981-991 https://doi.org/10.1002/jctb.4603.

[155] M.F. Musila, S.F. Dossaji, J.M. Nguta, C.W. Lukhoba, J.M. Munyao, In vivo antimalarial activity, toxicity and phytochemical screening of selected antimalarial plants, Journal of Ethnopharmacology, 146 (2013) 557-561 https://doi.org/10.1016/j.jep.2013.01.023.

[156] T. Kim, C. Jo, H.-S. Kim, Y.-M. Park, Y.-X. Wu, J.-H. Cho, T.H. Kim, Chemical constituents from Ainsliaea acerifolia as potential anti-obesity agents, Phytochemistry Letters, 16 (2016) 146-151 https://doi.org/10.1016/j.phytol.2016.04.005.

[157] Y. Zhu, L.-X. Zhang, Y. Zhao, G.-D. Huang, Unusual sesquiterpene lactones with a new carbon skeleton and new acetylenes from Ajania przewalskii, Food Chemistry, 118 (2010) 228-238 https://doi.org/10.1016/j.foodchem.2009.04.112.

[158] M. Ibrahim, T. Farooq, N. Hussain, A. Hussain, T. Gulzar, I. Hussain, M.S. Akash, F.S. Rehmani, Acetyl and butyryl cholinesterase inhibitory sesquiterpene lactones from Amberboa ramosa, Chemistry Central Journal, 7 (2013) 116-116 https://doi.org/10.1186/1752-153x-7-116.

[159] N.d.S. Jimenez-Usuga, N. Malafronte, R. Cotugno, M. De Leo, E. Osorio, N. De Tommasi, New sesquiterpene lactones from Ambrosia cumanensis Kunth, Fitoterapia, 113 (2016) 170-174 https://doi.org/10.1016/j.fitote.2016.07.019.

[160] V. Saroglou, A. Karioti, A. Rancic, K. Dimas, C. Koukoulitsa, M. Zervou, H. Skaltsa, Sesquiterpene Lactones from Anthemis melanolepis and Their Antibacterial and Cytotoxic Activities. Prediction of Their Pharmacokinetic Profile, Journal of Natural Products, 73 (2010) 242-246 https://doi.org/10.1021/np9004129.

[161] M. Todorova, A. Trendafilova, K. Danova, D. Dimitrov, Phytochemical study of Anthemis rumelica (Velen.) Stoj. & Acht, Biochemical Systematics and Ecology, 39 (2011) 868-871 https://doi.org/10.1016/j.bse.2011.06.003.

[162] F.T. Gürağaç Dereli, M. Ilhan, E. Küpeli Akkol, Discovery of new antidepressant agents: In vivo study on Anthemis wiedemanniana Fisch. & Mey, Journal of Ethnopharmacology, 226 (2018) 11-16 https://doi.org/10.1016/j.jep.2018.07.019.

[163] D.H. Saoud, A. Jelassi, M.B. Hlila, M.B. Goudjil, S. Ladjel, H.B. Jannet, Biological activities of extracts and metabolites isolated from Anvillea radiata Coss. & Dur. (Asteraceae), South African Journal of Botany, 121 (2019) 386-393 https://doi.org/10.1016/j.sajb.2018.10.033.

[164] H.A. Priestap, A. Galvis, N. Rivero, V. Costantino, L.A. Lopez, M.A. Barbieri, Dehydroleucodine and dehydroparishin-B inhibit proliferation and motility of B16 melanoma cells, Phytochemistry Letters, 5 (2012) 581-585 https://doi.org/10.1016/j.phytol.2012.05.018.

[165] R.M. Ghalib, R. Hashim, O. Sulaiman, S.H. Mehdi, A. Valkonen, K. Rissanen, S.R. Trifunović, M.B. Khadeer Ahamed, A.M.S.A. Majid, F. Kawamura, A novel caryophyllene type sesquiterpene lactone from Asparagus falcatus (Linn.); Structure elucidation and anti-angiogenic activity on HUVECs, European Journal of Medicinal Chemistry, 47 (2012) 601-607 https://doi.org/10.1016/j.ejmech.2011.10.037.

[166] J. Zhang, X. Hu, W. Gao, Z. Qu, H. Guo, Z. Liu, C. Liu, Pharmacokinetic study on costunolide and dehydrocostuslactone after oral administration of traditional medicine Aucklandia lappa Decne.

by LC/MS/MS, Journal of Ethnopharmacology, 151 (2014) 191-197 https://doi.org/10.1016/j.jep.2013.10.024.

[167] V. Gogineni, M.A. Nael, F. León, M.J. Núñez, S.J. Cutler, Computationally aided stereochemical assignment of undescribed bisabolenes from Calea urticifolia, Phytochemistry, 157 (2019) 145-150 https://doi.org/10.1016/j.phytochem.2018.10.022.

[168] H.-b. Wu, H.-b. Wu, W.-s. Wang, T.-t. Liu, M.-g. Qi, J.-c. Feng, X.-y. Li, Y. Liu, Insecticidal activity of sesquiterpene lactones and monoterpenoid from the fruits of Carpesium abrotanoides, Industrial Crops and Products, 92 (2016) 77-83 https://doi.org/10.1016/j.indcrop.2016.07.046.

[169] Q.-L. Hu, P.-Q. Wu, Y.-H. Liu, F.-M. Qi, C.-X. Yu, Y. Zhao, Y.-F. Yu, D.-Q. Fei, Z.-X. Zhang, Three new sesquiterpene lactones from Carpesium abrotanoides, Phytochemistry Letters, 27 (2018) 154-159 https://doi.org/10.1016/j.phytol.2018.07.010.

[170] D.-D. Xu, Y. Yan, C.-X. Jiang, J.-J. Liang, H.-F. Li, Q.-X. Wu, Y. Zhu, Sesquiterpenes and diterpenes with cytotoxic activities from the aerial parts of Carpesium humile, Fitoterapia, 128 (2018) 50-56 https://doi.org/10.1016/j.fitote.2018.04.015.

[171] J.-p. Zhang, X.-k. Xu, J. Ye, Y.-x. Yang, S. Gao, H.-l. Li, W.-d. Zhang, Three new sesquiterpene lactone dimers from Carpesium macrocephalum, Fitoterapia, 110 (2016) 72-76 https://doi.org/10.1016/j.fitote.2016.02.013.

[172] T. Milošević Ifantis, S. Solujić, D. Pavlović-Muratspahić, H. Skaltsa, Secondary metabolites from the aerial parts of Centaurea pannonica (Heuff.) Simonk. from Serbia and their chemotaxonomic importance, Phytochemistry, 94 (2013) 159-170 https://doi.org/10.1016/j.phytochem.2013.05.014.

[173] I. Komala, T. Ito, F. Nagashima, Y. Yagi, M. Kawahata, K. Yamaguchi, Y. Asakawa, Zierane sesquiterpene lactone, cembrane and fusicoccane diterpenoids, from the Tahitian liverwort Chandonanthus hirtellus, Phytochemistry, 71 (2010) 1387-1394 https://doi.org/10.1016/j.phytochem.2010.04.023.

[174] F. Wang, D.-S. Zhou, G.-Z. Wei, F.-C. Ren, J.-K. Liu, Chlorantholides A–F, eudesmane-type sesquiterpene lactones from Chloranthus elatior, Phytochemistry, 77 (2012) 312-317 https://doi.org/10.1016/j.phytochem.2012.02.008.

[175] J. Fu, S. Wang, H. Lu, J. Ma, X. Ke, T. Liu, Y. Luo, In vitro inhibitory effects of terpenoids from Chloranthus multistachys on epithelial–mesenchymal transition via down-regulation of Runx2 activation in human breast cancer, Phytomedicine, 22 (2015) 165-172 https://doi.org/10.1016/j.phymed.2014.11.010.

[176] X.-H. Li, H. Yan, W. Ni, X.-J. Qin, Q. Zhao, Z.-Q. Ji, H.-Y. Liu, Antifungal sesquiterpenoids from Chloranthus japonicus, Phytochemistry Letters, 15 (2016) 199-203 https://doi.org/10.1016/j.phytol.2016.01.005.

[177] F. Ayaz, N. Küçükboyacı, N. Gören, İ. Çalış, Ş. Aydınlık, E. Ulukaya, H. Duman, M.I. Choudhary, Bioassay-guided isolation of cytotoxic compounds from Chrysophthalmum montanum (DC.) Boiss, Food and Chemical Toxicology, 125 (2019) 10-20 https://doi.org/10.1016/j.fct.2018.12.029.

[178] L. Filippo D'Antuono, F. Ferioli, M.A. Manco, The impact of sesquiterpene lactones and phenolics on sensory attributes: An investigation of a curly endive and escarole germplasm collection, Food Chemistry, 199 (2016) 238-245 https://doi.org/10.1016/j.foodchem.2015.12.002.

[179] S.A. Mansour, R.M. Ibrahim, S.E. El-Gengaihi, Insecticidal activity of chicory (Cichorium intybus L.) extracts against two dipterous insect-disease vectors: Mosquito and housefly, Industrial Crops and Products, 54 (2014) 192-202 https://doi.org/10.1016/j.indcrop.2014.01.011.

[180] C. Barda, A. Ciric, M. Soković, M. Tsoukalas, H. Skaltsa, Phytochemical investigation of Crepis incana Sm. (Asteraceae) endemic to southern Greece, Biochemical Systematics and Ecology, 80 (2018) 59-62 https://doi.org/10.1016/j.bse.2018.06.009.

[181] U.U. Tambewagh, A.D. Kandhare, V.S. Honmore, P.P. Kadam, V.M. Khedkar, S.L. Bodhankar, S.R. Rojatkar, Anti-inflammatory and antioxidant potential of Guaianolide isolated from Cyathocline purpurea: Role of COX-2 inhibition, International Immunopharmacology, 52 (2017) 110-118 https://doi.org/10.1016/j.intimp.2017.09.001.

[182] M.A. Farag, M.F. Elsebai, A.R. Khattab, Metabolome based classification of artichoke leaf: A prospect for phyto-equivalency of its different leaf origins and commercial preparations, Journal of Pharmaceutical and Biomedical Analysis, 158 (2018) 151-159 https://doi.org/10.1016/j.jpba.2018.05.046.

[183] C. Paz, V. Burgos, A. Iturra, R. Rebolledo, L. Ortiz, R. Baggio, J. Becerra, C.L. Cespedes-Acuña, Assessment of insecticidal responses of extracts and compounds of Drimys winteri, Lobelia tupa, Viola portalesia and Vestia foetida against the granary weevil Sitophilus granarius, Industrial Crops and Products, 122 (2018) 232-238 https://doi.org/10.1016/j.indcrop.2018.06.009.

[184] Z.-N. Wu, Y.-B. Zhang, N.-H. Chen, M.-J. Li, M.-M. Li, W. Tang, L. Zhuang, Y.-L. Li, G.-C. Wang, Sesquiterpene lactones from Elephantopus mollis and their anti-inflammatory activities, Phytochemistry, 137 (2017) 81-86 https://doi.org/10.1016/j.phytochem.2017.01.020.

[185] A. Derksen, J. Kühn, W. Hafezi, J. Sendker, C. Ehrhardt, S. Ludwig, A. Hensel, Antiviral activity of hydroalcoholic extract from Eupatorium perfoliatum L. against the attachment of influenza A virus, Journal of Ethnopharmacology, 188 (2016) 144-152 https://doi.org/10.1016/j.jep.2016.05.016.

[186] M. Maas, A.M. Deters, A. Hensel, Anti-inflammatory activity of Eupatorium perfoliatum L. extracts, eupafolin, and dimeric guaianolide via iNOS inhibitory activity and modulation of inflammation-related cytokines and chemokines, Journal of Ethnopharmacology, 137 (2011) 371-381 https://doi.org/10.1016/j.jep.2011.05.040.

[187] P.E. Ordóñez, C.L. Quave, W.F. Reynolds, K.I. Varughese, B. Berry, P.J. Breen, O. Malagón, M.S. Smeltzer, C.M. Compadre, Sesquiterpene lactones from Gynoxys verrucosa and their anti-MRSA activity, Journal of Ethnopharmacology, 137 (2011) 1055-1059 https://doi.org/10.1016/j.jep.2011.07.012.

[188] P.E. Ordóñez, K.K. Sharma, L.M. Bystrom, M.A. Alas, R.G. Enriquez, O. Malagón, D.E. Jones, M.L. Guzman, C.M. Compadre, Dehydroleucodine, a Sesquiterpene Lactone from Gynoxys verrucosa, Demonstrates Cytotoxic Activity against Human Leukemia Cells, Journal of Natural Products, 79 (2016) 691-696 https://doi.org/10.1021/acs.jnatprod.5b00383.

[189] J.R. Prasifka, O. Spring, J. Conrad, L.W. Cook, D.E. Palmquist, M.E. Foley, Sesquiterpene Lactone Composition of Wild and Cultivated Sunflowers and Biological Activity against an Insect Pest, Journal of Agricultural and Food Chemistry, 63 (2015) 4042-4049 https://doi.org/10.1021/acs.jafc.5b00362.

[190] X. Yuan, M. Cheng, M. Gao, R. Zhuo, L. Zhang, H. Xiao, Cytotoxic constituents from the leaves of Jerusalem artichoke (Helianthus tuberosus L.) and their structure–activity relationships, Phytochemistry Letters, 6 (2013) 21-25 https://doi.org/10.1016/j.phytol.2012.10.007.

[191] V. Milutinović, M. Niketić, A. Krunić, D. Nikolić, M. Petković, L. Ušjak, S. Petrović, Sesquiterpene lactones from the methanol extracts of twenty-eight Hieracium species from the Balkan Peninsula and their chemosystematic significance, Phytochemistry, 154 (2018) 19-30 https://doi.org/10.1016/j.phytochem.2018.06.008.

[192] H.-x. Liu, Q.-y. Sun, F.-m. Yang, F.-w. Zhao, Y.-h. Wang, C.-l. Long, A new sesquiterpene lactone from Hosta ensata, Chemistry of Natural Compounds, 48 (2012) 580-582 https://doi.org/10.1007/s10600-012-0316-z.

[193] A.M. Fortuna, Z.N. Juárez, H. Bach, A. Nematallah, Y. Av-Gay, E. Sánchez-Arreola, C.A.N. Catalán, S. Turbay, L.R. Hernández, Antimicrobial activities of sesquiterpene lactones and inositol derivatives from Hymenoxys robusta, Phytochemistry, 72 (2011) 2413-2418 https://doi.org/10.1016/j.phytochem.2011.09.001.

[194] D. Huang, H. Deng, W. Chen, G. Huang, C. Chen, L. Sun, Four new sesquiterpene lactones from the stem bark of Illicium burmanicum, Fitoterapia, 92 (2014) 194-199 https://doi.org/10.1016/j.fitote.2013.11.001.

[195] P.-Y. Zhuang, G.-J. Zhang, X.-J. Wang, Y. Zhang, S.-S. Yu, Sesquiterpenes and prenylated C6–C3 compounds from the stems of Illicium henryi, Chinese Chemical Letters, 26 (2015) 1538-1541 https://doi.org/10.1016/j.cclet.2015.09.007.

[196] L. Fang, X.-j. Wang, S.-g. Ma, S.-s. Yu, A new sesquiterpene lactone and a new aromatic glycoside from Illicium difengpi, Acta Pharmaceutica Sinica B, 1 (2011) 178-183 https://doi.org/10.1016/j.apsb.2011.08.002.

[197] Y. Ren, U.M. Acuña, F. Jiménez, R. García, M. Mejía, H. Chai, J.C. Gallucci, N.R. Farnsworth, D.D. Soejarto, E.J. Carcache de Blanco, A.D. Kinghorn, Cytotoxic and NF-κB inhibitory sesquiterpene lactones from Piptocoma rufescens, Tetrahedron, 68 (2012) 2671-2678 https://doi.org/10.1016/j.tet.2012.01.061.

[198] W. Cai, J.-Y. Zhang, L.-Y. Dong, P.-H. Yin, C.-G. Wang, J.-Q. Lu, H.-G. Zhang, Identification of the metabolites of Ixerin Z from Ixeris sonchifolia Hance in rats by HPLC–LTQ-Orbitrap mass spectrometry, Journal of Pharmaceutical and Biomedical Analysis, 107 (2015) 290-297 https://doi.org/10.1016/j.jpba.2015.01.004.

[199] K. Michalska, W. Kisiel, A new guaianolide glucoside from aerial parts of Lactuca aculeata, Phytochemistry Letters, 5 (2012) 301-303 https://doi.org/10.1016/j.phytol.2012.02.006.

[200] Y.-F. Han, G.-X. Cao, X.-J. Gao, M. Xia, Isolation and characterisation of the sesquiterpene lactones from Lactuca sativa L var. anagustata, Food Chemistry, 120 (2010) 1083-1088 https://doi.org/10.1016/j.foodchem.2009.11.056.

[201] F. Mai, M.A. Glomb, Structural and Sensory Characterization of Novel Sesquiterpene Lactones from Iceberg Lettuce, Journal of Agricultural and Food Chemistry, 64 (2016) 295-301 https://doi.org/10.1021/acs.jafc.5b05128.

[202] V. Popović, A. Heyerick, S. Petrović, S. Van Calenbergh, I. Karalić, M. Niketić, D. Deforce, Sesquiterpene lactones from the extracts of two Balkan endemic Laserpitium species and their cytotoxic activity, Phytochemistry, 87 (2013) 102-111 https://doi.org/10.1016/j.phytochem.2012.11.011.

[203] M. Saleem, S. Parveen, N. Riaz, M.N. Tahir, M. Ashraf, I. Afzal, M.S. Ali, A. Malik, A. Jabbar, New bioactive natural products from Launaea nudicaulis, Phytochemistry Letters, 5 (2012) 793-799 https://doi.org/10.1016/j.phytol.2012.09.004.

[204] E. Julianti, K.H. Jang, S. Lee, D. Lee, W. Mar, K.-B. Oh, J. Shin, Sesquiterpenes from the leaves of Laurus nobilis L, Phytochemistry, 80 (2012) 70-76 https://doi.org/10.1016/j.phytochem.2012.05.013.

[205] M.R. de Souza, C.A. de Paula, M.L. Pereira de Resende, A. Grabe-Guimarães, J.D. de Souza Filho, D.A. Saúde-Guimarães, Pharmacological basis for use of Lychnophora trichocarpha in gouty arthritis: Anti-hyperuricemic and anti-inflammatory effects of its extract, fraction and constituents, Journal of Ethnopharmacology, 142 (2012) 845-850 https://doi.org/10.1016/j.jep.2012.06.012.

[206] E. Ríos V, A. León, M.I. Chávez, Y. Torres, M.T. Ramírez-Apan, R.A. Toscano, Á.E. Bravo-Monzón, F.J. Espinosa-García, G. Delgado, Sesquiterpene lactones from Mikania micrantha and Mikaniacordifolia and their cytotoxic and anti-inflammatory evaluation, Fitoterapia, 94 (2014) 155-163 https://doi.org/10.1016/j.fitote.2014.02.006.

[207] N.A. Jani, H.M. Sirat, F. Ahmad, S.A. Abed, N.I. Aminudin, Chemical constituents of the stems of Neolitsea kedahensis Gamble, Phytochemistry Letters, 26 (2018) 12-15 https://doi.org/10.1016/j.phytol.2018.05.001.

[208] B. Walshe-Roussel, C. Choueiri, A. Saleem, M. Asim, F. Caal, V. Cal, M.O. Rojas, T. Pesek, T. Durst, J.T. Arnason, Potent anti-inflammatory activity of sesquiterpene lactones from Neurolaena lobata (L.) R. Br. ex Cass., a Q'eqchi' Maya traditional medicine, Phytochemistry, 92 (2013) 122-127 https://doi.org/10.1016/j.phytochem.2013.05.004.

[209] Á.M. Móricz, D. Krüzselyi, Á. Alberti, A. Darcsi, G. Horváth, P. Csontos, S. Béni, P.G. Ott, Layer chromatography-bioassays directed screening and identification of antibacterial compounds from Scotch thistle, Journal of Chromatography A, 1524 (2017) 266-272 https://doi.org/10.1016/j.chroma.2017.09.062.

[210] X.-Y. Pang, Y.-X. Li, Y. Gong, Y. Yan, H.-F. Li, Y. Zhu, Sesquiterpenes from the whole plants of Parasenecio roborowskii, Fitoterapia, 116 (2017) 24-33 https://doi.org/10.1016/j.fitote.2016.10.010.

[211] Y.S. Hernández, L.B. Sánchez, M.M.G. Bedia, L.T. Gómez, E.J. Rodríguez, H.M.G.S. Miguel, D.G. Mosquera, L.P. García, L. Dhooghe, M. Theunis, L. Pieters, S. Apers, Determination of parthenin in Parthenium hysterophorus L. by means of HPLC-UV: Method development and validation, Phytochemistry Letters, 4 (2011) 134-137 https://doi.org/10.1016/j.phytol.2011.02.001.

[212] G. Odonne, G. Herbette, V. Eparvier, G. Bourdy, R. Rojas, M. Sauvain, D. Stien, Antileishmanial sesquiterpene lactones from Pseudelephantopus spicatus, a traditional remedy from the Chayahuita Amerindians (Peru). Part III, Journal of Ethnopharmacology, 137 (2011) 875-879 https://doi.org/10.1016/j.jep.2011.07.008.

[213] C. Girardi, N. Fabre, L. Paloque, A.P. Ramadani, F. Benoit-Vical, G. González-Aspajo, M. Haddad, E. Rengifo, V. Jullian, Evaluation of antiplasmodial and antileishmanial activities of herbal medicine Pseudelephantopus spiralis (Less.) Cronquist and isolated hirsutinolide-type sesquiterpenoids, Journal of Ethnopharmacology, 170 (2015) 167-174 https://doi.org/10.1016/j.jep.2015.05.014.

[214] N.d.S. Jimenez Usuga, N. Malafronte, E.J.O. Durango, A. Braca, N. De Tommasi, Phytochemical investigation of Pseudelephantopus spiralis (Less.) Cronquist, Phytochemistry Letters, 15 (2016) 256-259 https://doi.org/10.1016/j.phytol.2016.02.013.

[215] L. Pan, D.D. Lantvit, S. Riswan, L.B.S. Kardono, H.-B. Chai, E.J. Carcache de Blanco, N.R. Farnsworth, D.D. Soejarto, S.M. Swanson, A.D. Kinghorn, Bioactivity-guided isolation of cytotoxic sesquiterpenes of Rolandra fruticosa, Phytochemistry, 71 (2010) 635-640 https://doi.org/10.1016/j.phytochem.2010.01.012.

[216] L.C. Fonseca, S.S. Dadarkar, A.S. Lobo, A.C. Suthar, V.S. Chauhan, S. Chandrababu, S.D. Sharma, N.M. Dagia, M. Padigaru, 7-hydroxyfrullanolide, a sesquiterpene lactone, inhibits pro-inflammatory cytokine production from immune cells and is orally efficacious in animal models of inflammation, European Journal of Pharmacology, 644 (2010) 220-229 https://doi.org/10.1016/j.ejphar.2010.06.052.

[217] W. Xiao, X. Li, N. Li, M. Bolati, X. Wang, X. Jia, Y. Zhao, Sesquiterpene lactones from Saussurea involucrata, Fitoterapia, 82 (2011) 983-987 https://doi.org/10.1016/j.fitote.2011.05.015.

[218] L. Ruiz-Vásquez, M. Reina, M. López-Rodríguez, C. Giménez, R. Cabrera, P. Cuadra, V. Fajardo, A. González-Coloma, Sesquiterpenes, flavonoids, shikimic acid derivatives and pyrrolizidine alkaloids from Senecio kingii Hook, Phytochemistry, 117 (2015) 245-253 https://doi.org/10.1016/j.phytochem.2015.06.019.

[219] C. De Ford, J.L. Ulloa, C.A.N. Catalán, A. Grau, V.S. Martino, L.V. Muschietti, I. Merfort, The sesquiterpene lactone polymatin B from Smallanthus sonchifolius induces different cell death

mechanisms in three cancer cell lines, Phytochemistry, 117 (2015) 332-339 https://doi.org/10.1016/j.phytochem.2015.06.020.

[220] Z. Xia, J. Yao, J. Liang, Two new sesquiterpene lactones from Sonchus arvensis, Chemistry of Natural Compounds, 48 (2012) 47-50 https://doi.org/10.1007/s10600-012-0155-y.

[221] Z. Xia, W. Qu, H. Lu, J. Fu, Y. Ren, J. Liang, Sesquiterpene lactones from Sonchus arvensis L. and their antibacterial activity against Streptococcus mutans ATCC 25175, Fitoterapia, 81 (2010) 424-428 https://doi.org/10.1016/j.fitote.2009.12.001.

[222] W. Sangsopha, R. Lekphrom, S. Kanokmedhakul, K. Kanokmedhakul, Cytotoxic and antimalarial constituents from aerial parts of Sphaeranthus indicus, Phytochemistry Letters, 17 (2016) 278-281 https://doi.org/10.1016/j.phytol.2016.08.001.

[223] J. Qu, S. Deng, L. Li, Y. Liu, Y. Li, S. Ma, X. Chen, S. Yu, Cytotoxic dimeric xanthanolides from fruits of Xanthium chinense, Phytochemistry, 132 (2016) 115-122 https://doi.org/10.1016/j.phytochem.2016.10.006.

[224] W.D. Li, Y. Wu, L. Zhang, L.G. Yan, F.Z. Yin, J.S. Ruan, Z.P. Chen, G.M. Yang, C.P. Yan, D. Zhao, Y. Lu, B.C. Cai, Characterization of xanthatin: Anticancer properties and mechanisms of inhibited murine melanoma in vitro and in vivo, Phytomedicine, 20 (2013) 865-873 https://doi.org/10.1016/j.phymed.2013.03.006.

[225] A. Altemimi, N. Lakhssassi, A. Baharlouei, D.G. Watson, D.A. Lightfoot, Phytochemicals: Extraction, Isolation, and Identification of Bioactive Compounds from Plant Extracts, Plants, 6 (2017) 42 https://doi.org/10.3390/plants6040042

[226] J. Azmir, I.S.M. Zaidul, M.M. Rahman, K.M. Sharif, A. Mohamed, F. Sahena, M.H.A. Jahurul, K. Ghafoor, N.A.N. Norulaini, A.K.M. Omar, Techniques for extraction of bioactive compounds from plant materials: A review, Journal of Food Engineering, 117 (2013) 426-436 https://doi.org/10.1016/j.jfoodeng.2013.01.014.

[227] T. Belwal, S.M. Ezzat, L. Rastrelli, I.D. Bhatt, M. Daglia, A. Baldi, H.P. Devkota, I.E. Orhan, J.K. Patra, G. Das, C. Anandharamakrishnan, L. Gomez-Gomez, S.F. Nabavi, S.M. Nabavi, A.G. Atanasov, A critical analysis of extraction techniques used for botanicals: Trends, priorities, industrial uses and optimization strategies, TrAC Trends in Analytical Chemistry, 100 (2018) 82-102 https://doi.org/10.1016/j.trac.2017.12.018.

[228] S.C. Mandal, V. Mandal, A.K. Das, Chapter 6 - Classification of Extraction Methods, in: S.C. Mandal, V. Mandal, A.K. Das (Eds.) Essentials of Botanical Extraction, Academic Press, Boston, 2015, pp. 83-136 ISBN: 978-0-12-802325-9.

[229] C.G. Pereira, M.A.A. Meireles, Supercritical Fluid Extraction of Bioactive Compounds: Fundamentals, Applications and Economic Perspectives, Food and Bioprocess Technology, 3 (2010) 340-372 https://doi.org/10.1007/s11947-009-0263-2.

[230] L. Martín, L.F. Julio, J. Burillo, J. Sanz, A.M. Mainar, A. González-Coloma, Comparative chemistry and insect antifeedant action of traditional (Clevenger and Soxhlet) and supercritical extracts (CO2) of two cultivated wormwood (Artemisia absinthium L.) populations, Industrial Crops and Products, 34 (2011) 1615-1621 https://doi.org/10.1016/j.indcrop.2011.06.006.

[231] F. Ferioli, L.F. D'Antuono, An update procedure for an effective and simultaneous extraction of sesquiterpene lactones and phenolics from chicory, Food Chemistry, 135 (2012) 243-250 https://doi.org/10.1016/j.foodchem.2012.04.079.

[232] A. Mustafa, C. Turner, Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review, Analytica Chimica Acta, 703 (2011) 8-18 https://doi.org/10.1016/j.aca.2011.07.018.

[233] M. Herrero, A.d.P. Sánchez-Camargo, A. Cifuentes, E. Ibáñez, 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, 71 (2015) 26-38 https://doi.org/10.1016/j.trac.2015.01.018.

[234] A. Ollivier, R. Grougnet, X. Cachet, D. Meriane, J. Ardisson, S. Boutefnouchet, B. Deguin, Large scale purification of the SERCA inhibitor Thapsigargin from Thapsia garganica L. roots using centrifugal partition chromatography, Journal of Chromatography B, 926 (2013) 16-20 https://doi.org/10.1016/j.jchromb.2013.02.015.

[235] E. Destandau, M.A. Boukhris, S. Zubrzycki, M. Akssira, L.E. Rhaffari, C. Elfakir, Centrifugal partition chromatography elution gradient for isolation of sesquiterpene lactones and flavonoids from Anvillea radiata, Journal of Chromatography B, 985 (2015) 29-37 https://doi.org/10.1016/j.jchromb.2015.01.019.

[236] G. Graziani, R. Ferracane, P. Sambo, S. Santagata, C. Nicoletto, V. Fogliano, Profiling chicory sesquiterpene lactones by high resolution mass spectrometry, Food Research International, 67 (2015) 193-198 https://doi.org/10.1016/j.foodres.2014.11.021.

[237] M. Romero, M. Zanuy, E. Rosell, M. Cascante, J. Piulats, M. Font-Bardia, J. Balzarini, E. De Clerq, M.D. Pujol, Optimization of xanthatin extraction from Xanthium spinosum L. and its cytotoxic, anti-angiogenesis and antiviral properties, European Journal of Medicinal Chemistry, 90 (2015) 491-496 https://doi.org/10.1016/j.ejmech.2014.11.060.

[238] M. Kim, K. Song, Y.S. Kim, Alantolactone improves palmitate-induced glucose intolerance and inflammation in both lean and obese states in vitro: Adipocyte and adipocyte-macrophage co-culture system, International Immunopharmacology, 49 (2017) 187-194 https://doi.org/10.1016/j.intimp.2017.05.037.

[239] J. Li, Y. Yoshida, M. Kurita, T. Usuki, Cynaropicrin and inhibition of NF-κB activation: A structure activity relationship study, Bioorganic & Medicinal Chemistry Letters, 29 (2019) 1518-1521 https://doi.org/10.1016/j.bmcl.2019.04.004.

[240] A. Scavo, G. Pandino, A. Restuccia, G. Mauromicale, Leaf extracts of cultivated cardoon as potential bioherbicide, Scientia Horticulturae, 261 (2020) 109024 https://doi.org/10.1016/j.scienta.2019.109024.

[241] L. Wang, C.L. Weller, Recent advances in extraction of nutraceuticals from plants, Trends in Food Science & Technology, 17 (2006) 300-312 https://doi.org/10.1016/j.tifs.2005.12.004.

[242] C.S. Frederick, Sesquiterpene Lactones as Taxonomic Characters in the Asteraceae, Botanical Review, 48 (1982) 121-595 http://dx.doi.org/10.1007/BF02919190

[243] Z. Velez, M.A. Campinho, Â.R. Guerra, L. García, P. Ramos, O. Guerreiro, L. Felício, F. Schmitt, M. Duarte, Biological Characterization of Cynara cardunculus L. Methanolic Extracts: Antioxidant, Anti-proliferative, Anti-migratory and Anti-angiogenic Activities, Agriculture, 2 (2012) 472-492 https://doi.org/10.3390/agriculture2040472.

[244] C. Zidorn, Sesquiterpene lactones and their precursors as chemosystematic markers in the tribe Cichorieae of the Asteraceae, Phytochemistry, 69 (2008) 2270-2296 https://doi.org/10.1016/j.phytochem.2008.06.013.

[245] P.R. Bhattacharyya, N.C. Barua, A.C. Ghosh, Cynaropicrin from Tricholepis glaberrima: a potential insect feeding deterrent compound, Industrial Crops and Products, 4 (1995) 291-294 https://doi.org/10.1016/0926-6690(95)00044-5.

[246] S. Zimmermann, M. Oufir, A. Leroux, R.L. Krauth-Siegel, K. Becker, M. Kaiser, R. Brun, M. Hamburger, M. Adams, Cynaropicrin targets the trypanothione redox system in Trypanosoma brucei, Bioorganic & Medicinal Chemistry, 21 (2013) 7202-7209 https://doi.org/10.1016/j.bmc.2013.08.052.

[247] M.A. Margulis, I.M. Margulis, Calorimetric method for measurement of acoustic power absorbed in a volume of a liquid, Ultrasonics Sonochemistry, 10 (2003) 343-345 https://doi.org/10.1016/S1350-4177(03)00100-7.

[248] R.S.o. Chemistry, www.chemspider.com/Chemical-Structure.106409.html, accessed in 15/08/2017

[249] C.M. Hansen, Hansen solubility parameters: a user's handbook, CRC press, 2002 ISBN: 9780849372483.

[250] K. Bundeesomchok, A. Filly, N. Rakotomanomana, P. Panichayupakaranant, F. Chemat, Extraction of α-mangostin from Garcinia mangostana L. using alternative solvents: Computational predictive and experimental studies, LWT-Food Science and Technology, 65 (2016) 297-303 https://doi.org/10.1016/j.lwt.2015.08.036.

[251] A. Filly, A.-S. Fabiano-Tixier, Y. Lemasson, C. Roy, X. Fernandez, F. Chemat, Extraction of aroma compounds in blackcurrant buds by alternative solvents: Theoretical and experimental solubility study, Comptes Rendus Chimie, 17 (2014) 1268-1275 https://doi.org/10.1016/j.crci.2014.03.013.

[252] S. Hemwimon, P. Pavasant, A. Shotipruk, Microwave-assisted extraction of antioxidative anthraquinones from roots of Morinda citrifolia, Separation and Purification Technology, 54 (2007) 44-50 https://doi.org/10.1016/j.seppur.2006.08.014.

[253] N. Azwanida, A review on the extraction methods use in medicinal plants, principle, strength and limitation, Med Aromat Plants, 4 (2015) 2167-0412.1000196 https://doi.org/10.4172/2167-0412.1000196.

[254] C. Proestos, M. Komaitis, Application of microwave-assisted extraction to the fast extraction of plant phenolic compounds, LWT - Food Science and Technology, 41 (2008) 652-659 https://doi.org/10.1016/j.lwt.2007.04.013.

[255] K. Yasui, Chapter 3 - Dynamics of Acoustic Bubbles, in: Sonochemistry and the Acoustic Bubble, Elsevier, Amsterdam, 2015, pp. 41-83 ISBN: 978-0-12-801530-8.

[256] H. Li, L. Pordesimo, J. Weiss, High intensity ultrasound-assisted extraction of oil from soybeans, Food Research International, 37 (2004) 731-738 https://doi.org/10.1016/j.foodres.2004.02.016.

[257] A. Shotipruk, P.B. Kaufman, H.Y. Wang, Feasibility Study of Repeated Harvesting of Menthol from Biologically Viable Menthaxpiperata Using Ultrasonic Extraction, Biotechnology Progress, 17 (2001) 924-928 https://doi.org/10.1021/bp010074u.

[258] K. Eljounaidi, C. Comino, A. Moglia, K. Cankar, A. Genre, A. Hehn, F. Bourgaud, J. Beekwilder, S. Lanteri, Accumulation of cynaropicrin in globe artichoke and localization of enzymes involved in its biosynthesis, Plant Science, 239 (2015) 128-136 https://doi.org/10.1016/j.plantsci.2015.07.020.

[259] H. Sovová, L. Opletal, M. Sajfrtová, M. Bártlová, Supercritical fluid extraction of cynaropicrin and 20-hydroxyecdysone from Leuzea carthamoides DC, Journal of Separation Science, 31 (2008) 1387-1392 https://doi.org/10.1002/jssc.200700496.

[260] J. Gominho, M.D. Curt, A. Lourenço, J. Fernández, H. Pereira, Cynara cardunculus L. as a biomass and multi-purpose crop: A review of 30 years of research, Biomass and Bioenergy, 109 (2018) 257-275 https://doi.org/10.1016/j.biombioe.2018.01.001.

[261] T. Brás, O. Guerreiro, M.F. Duarte, L.A. Neves, Impact of extraction parameters and concentration by nanofiltration on the recovery of phenolic compounds from Cynara cardunculus var. altilis: Assessment of antioxidant activity, Industrial Crops and Products, 67 (2015) 137-142 https://doi.org/10.1016/j.indcrop.2015.01.005.

[262] P.A.B. Ramos, A.M. Ferro, M.M. Oliveira, S. Gonçalves, C.S.R. Freire, A.J.D. Silvestre, M.F. Duarte, Biosynthesis and bioactivity of Cynara cardunculus L. guaianolides and hydroxycinnamic acids: a genomic, biochemical and health-promoting perspective, Phytochemistry Reviews, (2019) 18, 495–526 https://doi.org/10.1007/s11101-019-09604-4.

[263] P.A.B. Ramos, S.A.O. Santos, Â.R. Guerra, O. Guerreiro, C.S.R. Freire, S.M. Rocha, M.F. Duarte, A.J.D. Silvestre, Phenolic composition and antioxidant activity of different morphological parts of Cynara cardunculus L. var. altilis (DC), Industrial Crops and Products, 61 (2014) 460-471 https://doi.org/10.1016/j.indcrop.2014.07.042.

[264] C. Rial, P. Novaes, R.M. Varela, J.M. G. Molinillo, F.A. Macias, Phytotoxicity of Cardoon (Cynara cardunculus) Allelochemicals on Standard Target Species and Weeds, Journal of Agricultural and Food Chemistry, 62 (2014) 6699-6706 https://doi.org/10.1021/jf501976h.

[265] E. Espada-Bellido, M. Ferreiro-González, C. Carrera, M. Palma, C.G. Barroso, G.F. Barbero, Optimization of the ultrasound-assisted extraction of anthocyanins and total phenolic compounds in mulberry (Morus nigra) pulp, Food chemistry, 219 (2017) 23-32 https://doi.org/10.1016/j.foodchem.2016.09.122.

[266] S.A. Heleno, P. Diz, M. Prieto, L. Barros, A. Rodrigues, M.F. Barreiro, I.C. Ferreira, Optimization of ultrasound-assisted extraction to obtain mycosterols from Agaricus bisporus L. by response surface methodology and comparison with conventional Soxhlet extraction, Food chemistry, 197 (2016) 1054-1063 https://doi.org/10.1016/j.foodchem.2015.11.108.

[267] Y. Li, Y. Chen, H. Li, Recovery and purification of cholesterol from cholesterol-β-cyclodextrin inclusion complex using ultrasound-assisted extraction, Ultrasonics Sonochemistry, 34 (2017) 281-288 https://doi.org/10.1016/j.ultsonch.2016.05.032.

[268] J.P. Maran, B. Priya, N.A. Al-Dhabi, K. Ponmurugan, I.G. Moorthy, N. Sivarajasekar, Ultrasound assisted citric acid mediated pectin extraction from industrial waste of Musa balbisiana, Ultrasonics Sonochemistry, 35 (2017) 204-209 https://doi.org/10.1016/j.ultsonch.2016.09.019.

[269] I.G. Moorthy, J.P. Maran, S. Ilakya, S. Anitha, S.P. Sabarima, B. Priya, Ultrasound assisted extraction of pectin from waste Artocarpus heterophyllus fruit peel, Ultrasonics Sonochemistry, 34 (2017) 525-530 https://doi.org/10.1016/j.ultsonch.2016.06.015.

[270] S. Shirsath, S. Sonawane, P. Gogate, Intensification of extraction of natural products using ultrasonic irradiations—a review of current status, Chemical Engineering and Processing: Process Intensification, 53 (2012) 10-23 https://doi.org/10.1016/j.cep.2012.01.003.

[271] A. Tomsik, B. Pavlic, J. Vladic, M. Ramic, J. Brindza, S. Vidovic, Optimization of ultrasound-assisted extraction of bioactive compounds from wild garlic (Allium ursinum L.), Ultrasonics Sonochemistry, 29 (2016) 502-511 https://doi.org/10.1016/j.ultsonch.2015.11.005.

[272] S. Dey, V.K. Rathod, Ultrasound assisted extraction of β-carotene from Spirulina platensis, Ultrasonics Sonochemistry, 20 (2013) 271-276 https://doi.org/10.1016/j.ultsonch.2012.05.010.

[273] A.M. Goula, Ultrasound-assisted extraction of pomegranate seed oil – Kinetic modeling, Journal of Food Engineering, 117 (2013) 492-498 https://doi.org/10.1016/j.jfoodeng.2012.10.009.

[274] Z. Pan, W. Qu, H. Ma, G.G. Atungulu, T.H. McHugh, Continuous and pulsed ultrasound-assisted extractions of antioxidants from pomegranate peel, Ultrasonics Sonochemistry, 18 (2011) 1249-1257 https://doi.org/10.1016/j.ultsonch.2011.01.005.

[275] D.M. Patil, K.G. Akamanchi, Ultrasound-assisted rapid extraction and kinetic modelling of influential factors: Extraction of camptothecin from Nothapodytes nimmoniana plant, Ultrasonics Sonochemistry, 37 (2017) 582-591 https://doi.org/10.1016/j.ultsonch.2017.02.015.

[276] N.A. Al-Dhabi, K. Ponmurugan, P.M. Jeganathan, Development and validation of ultrasound-assisted solid-liquid extraction of phenolic compounds from waste spent coffee grounds, Ultrasonics Sonochemistry, 34 (2017) 206-213 https://doi.org/10.1016/j.ultsonch.2016.05.005.

[277] Z. Pan, W. Qu, H. Ma, G.G. Atungulu, T.H. McHugh, Continuous and pulsed ultrasound-assisted extractions of antioxidants from pomegranate peel, Ultrasonics Sonochemistry, 19 (2012) 365-372 https://doi.org/10.1016/j.ultsonch.2011.01.005.

[278] L.C. Hagenson, L.K. Doraiswamy, Comparison of the effects of ultrasound and mechanical agitation on a reacting solid-liquid system, Chemical Engineering Science, 53 (1998) 131-148 https://doi.org/10.1016/S0009-2509(97)00193-0.

[279] M. Vinatoru, An overview of the ultrasonically assisted extraction of bioactive principles from herbs, Ultrasonics Sonochemistry, 8 (2001) 303-313 https://doi.org/10.1016/S1350-4177(01)00071-2.

[280] S. Hemwimol, P. Pavasant, A. Shotipruk, Ultrasound-assisted extraction of anthraquinones from roots of Morinda citrifolia, Ultrasonics Sonochemistry, 13 (2006) 543-548 https://doi.org/10.1016/j.ultsonch.2005.09.009.

[281] M. Kazemi, R. Karim, H. Mirhosseini, A.A. Hamid, Optimization of pulsed ultrasound-assisted technique for extraction of phenolics from pomegranate peel of Malas variety: Punicalagin and hydroxybenzoic acids, Food chemistry, 206 (2016) 156-166 https://doi.org/10.1016/j.foodchem.2016.03.017.

[282] A. Zayed, A. Serag, M.A. Farag, Cynara cardunculus L.: Outgoing and potential trends of phytochemical, industrial, nutritive and medicinal merits, Journal of Functional Foods, 69 (2020) 103937 https://doi.org/10.1016/j.jff.2020.103937.

[283] I. Piscioneri, N. Sharma, G. Baviello, S. Orlandini, Promising industrial energy crop, Cynara cardunculus: a potential source for biomass production and alternative energy, Energy Conversion and Management, 41 (2000) 1091-1105 https://doi.org/10.1016/S0196-8904(99)00135-1.

[284] T. Brás, A.F.C. Paulino, L.A. Neves, J.G. Crespo, M.F. Duarte, Ultrasound assisted extraction of cynaropicrin from Cynara cardunculus leaves: Optimization using the response surface methodology and the effect of pulse mode, Industrial Crops and Products, 150 (2020) 112395 https://doi.org/10.1016/j.indcrop.2020.112395.

[285] L.K. Caesar, N.B. Cech, Synergy and antagonism in natural product extracts: when 1 + 1 does not equal 2, Natural Product Reports, 36 (2019) 869-888 https://doi.org/10.1039/c9np00011a.

[286] I. Khemakhem, O.D. Gargouri, A. Dhouib, M.A. Ayadi, M. Bouaziz, Oleuropein rich extract from olive leaves by combining microfiltration, ultrafiltration and nanofiltration, Separation and Purification Technology, 172 (2017) 310-317 https://doi.org/10.1016/j.seppur.2016.08.003.

[287] M. Almanasrah, C. Brazinha, M. Kallioinen, L.C. Duarte, L.B. Roseiro, R. Bogel-Lukasik, F. Carvalheiro, M. Mänttäri, J.G. Crespo, Nanofiltration and reverse osmosis as a platform for production of natural botanic extracts: The case study of carob by-products, Separation and Purification Technology, 149 (2015) 389-397 https://doi.org/10.1016/j.seppur.2015.06.008.

[288] H.M. Krieg, S.J. Modise, K. Keizer, H.W.J.P. Neomagus, Salt rejection in nanofiltration for single and binary salt mixtures in view of sulphate removal, Desalination, 171 (2005) 205-215 https://doi.org/10.1016/j.desal.2004.05.005.

[289] J.L. Cardador dos Santos, M.C. Fernandes, P.M.L. Lourenco, L.C. Duarte, F. Carvalheiro, J.G. Crespo, Removal of inhibitory compounds from olive stone auto-hydrolysis liquors by nanofiltration, Desalination and Water Treatment, 27 (2011) 90-96 https://doi.org/10.5004/dwt.2011.2063.

[290] Y.H. See Toh, X.X. Loh, K. Li, A. Bismarck, A.G. Livingston, In search of a standard method for the characterisation of organic solvent nanofiltration membranes, Journal of Membrane Science, 291 (2007) 120-125 https://doi.org/10.1016/j.memsci.2006.12.053.

[291] J. Wang, Z. Wang, Y. Liu, J. Wang, S. Wang, Surface modification of NF membrane with zwitterionic polymer to improve anti-biofouling property, Journal of Membrane Science, 514 (2016) 407-417 https://doi.org/10.1016/j.memsci.2016.05.014.

[292] E.M. Rundquist, Application and Evaluation of Organic Solvent Nanofiltration in Pharmaceutical Processing in: Department of Chemical Engineering and Chemical Technology Imperial College London 2013, https://spiral.imperial.ac.uk/bitstream/10044/1/39336/2/Rundquist-EM-2013-PhD-Thesis.pdf

[293] M. Gryta, J. Bastrzyk, D. Lech, Evaluation of fouling potential of nanofiltration membranes based on the dynamic contact angle measurements, Polish Journal of Chemical Technology, 14 (2012) 97-104 https://doi.org/10.2478/v10026-012-0091-4.

[294] S.B. Sigurdardóttir, M.S.M. Sueb, M. Pinelo, Membrane compaction, internal fouling, and membrane preconditioning as major factors affecting performance of solvent resistant nanofiltration membranes in methanol solutions, Separation and Purification Technology, 227 (2019) 115686 https://doi.org/10.1016/j.seppur.2019.115686.

[295] T. Brás, V. Guerra, I. Torrado, P. Lourenço, F. Carvalheiro, L.C. Duarte, L.A. Neves, Detoxification of hemicellulosic hydrolysates from extracted olive pomace by diananofiltration, Process Biochemistry, 49 (2014) 173-180 https://doi.org/10.1016/j.procbio.2013.09.017.

[296] DuraMem, https://duramem.evonik.com/product/peek-industrial/downloads/flyer%20duramem%20membranes%20technical%20information%2004-2018.pdf, accessed in 06/04/2020

[297] Matche, http://www.matche.com/EquipCost/, accessed in 01-04-2020

[298] Cynaropicrin, https://www.extrasynthese.com/cynaropicrin-1457.html?___SID=U, accessed in 06/04/2020

[299] C. Brazinha, M. Cadima, J.G. Crespo, Valorisation of spent coffee through membrane processing, Journal of Food Engineering, 149 (2015) 123-130 https://doi.org/10.1016/j.jfoodeng.2014.07.016.

[300] H. Sinno, S. Prakash, Complements and the wound healing cascade: an updated review, Plastic surgery international, 2013 (2013) https://doi.org/10.1155/2013/146764.

[301] K. Järbrink, G. Ni, H. Sönnergren, A. Schmidtchen, C. Pang, R. Bajpai, J. Car, The humanistic and economic burden of chronic wounds: a protocol for a systematic review, Systematic reviews, 6 (2017) 15-15 https://doi.org/10.1186/s13643-016-0400-8.

[302] S. Dhivya, V.V. Padma, E. Santhini, Wound dressings - a review, BioMedicine, 5 (2015) 22-22 https://doi.org/10.7603/s40681-015-0022-9.

[303] S. Ahmed, S. Ikram, Chitosan Based Scaffolds and Their Applications in Wound Healing, Achievements in the Life Sciences, 10 (2016) 27-37 https://doi.org/10.1016/j.als.2016.04.001.

[304] T. Dai, M. Tanaka, Y.-Y. Huang, M.R. Hamblin, Chitosan preparations for wounds and burns: antimicrobial and wound-healing effects, Expert review of anti-infective therapy, 9 (2011) 857-879 https://doi.org/10.1586/eri.11.59.

[305] H. Liu, C. Wang, C. Li, Y. Qin, Z. Wang, F. Yang, Z. Li, J. Wang, A functional chitosan-based hydrogel as a wound dressing and drug delivery system in the treatment of wound healing, Royal Society of Chemistry Advances, 8 (2018) 7533-7549 https://doi.org/10.1039/c7ra13510f.

[306] V. Patrulea, V. Ostafe, G. Borchard, O. Jordan, Chitosan as a starting material for wound healing applications, European Journal of Pharmaceutics and Biopharmaceutics, 97 (2015) 417-426 https://doi.org/10.1016/j.ejpb.2015.08.004.

[307] R. Jayakumar, M. Prabaharan, P.T. Sudheesh Kumar, S.V. Nair, H. Tamura, Biomaterials based on chitin and chitosan in wound dressing applications, Biotechnology Advances, 29 (2011) 322-337 https://doi.org/10.1016/j.biotechadv.2011.01.005.

[308] A.A. Muhammad, N.A.S. Pauzi, P. Arulselvan, F. Abas, S. Fakurazi, *In Vitro* Wound Healing Potential and Identification of Bioactive Compounds from *Moringa oleifera* Lam, BioMed Research International, 2013 (2013) 974580 https://doi.org/10.1155/2013/974580.

[309] S. Ghuman, B. Ncube, J.F. Finnie, L.J. McGaw, E. Mfotie Njoya, R.M. Coopoosamy, J. Van Staden, Antioxidant, anti-inflammatory and wound healing properties of medicinal plant extracts used to treat wounds and dermatological disorders, South African Journal of Botany, 126 (2019) 232-240 https://doi.org/10.1016/j.sajb.2019.07.013.

[310] B.P. George, T. Parimelazhagan, R. Chandran, Anti-inflammatory and wound healing properties of Rubus fairholmianus Gard. root—An in vivo study, Industrial Crops and Products, 54 (2014) 216-225 https://doi.org/10.1016/j.indcrop.2014.01.037.

[311] X. Jiang, Y. Chen, D. Liu, T. Shi, X. Cheng, W. He, Y. Li, B. Ryffel, S.G. Zheng, Y. Zheng, Secoeudesma sesquiterpenes lactone A alleviates inflammation and offers adjuvant protection in severe infection of carbapenem-resistant Klebsiella pneumoniae, Journal of Ethnopharmacology, 252 (2020) 112605 https://doi.org/10.1016/j.jep.2020.112605.

[312] Z.-l. Wu, J.-x. Wang, L.-p. Chen, H.-y. Dong, H.-l. Li, W.-d. Zhang, Five rare C32 sesquiterpene lactone dimers with anti-inflammation activity from Vladimiria souliei, Fitoterapia, 125 (2018) 117-122 https://doi.org/10.1016/j.fitote.2018.01.002.

[313] R. Sabel, A.S. Fronza, L.Z.B. Carrenho, A. Maes, M.L. Barros, L.A.E. Pollo, M.W. Biavatti, K. D'Herde, P. Vandenabeele, M.R.O. Kreuger, Anti-inflammatory activity of the sesquiterpene lactone diacethylpiptocarphol in dextransulfate sodium-induced colitis in mice, Journal of Ethnopharmacology, 245 (2019) 112186 https://doi.org/10.1016/j.jep.2019.112186.

[314] M.C. Recio, R.M. Giner, L. Uriburu, S. Máñez, M. Cerdá, J.R. De La Fuente, J.L. Ríos, In vivo activity of pseudoguaianolide sesquiterpene lactones in acute and chronic inflammation, Life Sciences, 66 (2000) 2509-2518 https://doi.org/10.1016/S0024-3205(00)00585-3.

[315] T. Brás, D. Rosa, A.C. Gonçalves, A.C. Gomes, C. Brazinha, L.A. Neves, M.F. Duarte, J.G. Crespo, Fractionation of Cynara cardunculus ethanolic extracts using diananofiltration, Separation and Purification Technology, (2020) ID SEPPUR-D-20-01874 R1.

[316] A.R.V. Ferreira, C.A.V. Torres, F. Freitas, C. Sevrin, C. Grandfils, M.A.M. Reis, V.D. Alves, I.M. Coelhoso, Development and characterization of bilayer films of FucoPol and chitosan, Carbohydrate Polymers, 147 (2016) 8-15 https://doi.org/10.1016/j.carbpol.2016.03.089.

[317] L. Greenspan, Humidity fixed points of binary saturated aqueous solutions, Journal of Research of the National Bureau of Standards, 81 (1977) 89-96 https://doi.org/10.6028/jres.081a.011.

[318] I.T. Meireles, C. Portugal, V.D. Alves, J.G. Crespo, I.M. Coelhoso, Impact of biopolymer purification on the structural characteristics and transport performance of composite polysaccharide membranes for pervaporation, Journal of Membrane Science, 493 (2015) 179-187 https://doi.org/10.1016/j.memsci.2015.07.011.

[319] I.O.f.S. (ISO), ISO 10993-5:2009 Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity, in, 2009.

[320] I. GmbH, Instructions µ-Plate 24 Well Black, https://ibidi.com/img/cms/products/labware/plates/P_824XX_Plate_24well/IN_824XX_24well.pdf, accessed in 10/2019

[321] I.F. Amaral, P.L. Granja, M.A. Barbosa, Chemical modification of chitosan by phosphorylation: an XPS, FT-IR and SEM study, Journal of Biomaterials Science, Polymer Edition, 16 (2005) 1575-1593 https://doi.org/10.1163/156856205774576736.

[322] U. Siripatrawan, W. Vitchayakitti, Improving functional properties of chitosan films as active food packaging by incorporating with propolis, Food Hydrocolloids, 61 (2016) 695-702 https://doi.org/10.1016/j.foodhyd.2016.06.001.

[323] L. Sun, J. Sun, L. Chen, P. Niu, X. Yang, Y. Guo, Preparation and characterization of chitosan film incorporated with thinned young apple polyphenols as an active packaging material, Carbohydrate Polymers, 163 (2017) 81-91 https://doi.org/10.1016/j.carbpol.2017.01.016.

[324] J. Coates, Interpretation of infrared spectra, a practical approach, Encyclopedia of analytical chemistry: applications, theory and instrumentation, (2006) https://doi.org/10.1002/9780470027318.a5606.

[325] R.A. Mauricio-Sánchez, R. Salazar, J.G. Luna-Bárcenas, A. Mendoza-Galván, FTIR spectroscopy studies on the spontaneous neutralization of chitosan acetate films by moisture conditioning, Vibrational Spectroscopy, 94 (2018) 1-6 https://doi.org/10.1016/j.vibspec.2017.10.005.

[326] S.M. Bishop, M. Walker, A.A. Rogers, W.Y.J. Chen, Importance of moisture balance at the wound-dressing interface, Journal of Wound Care, 12 (2003) 125-128 https://doi.org/10.12968/jowc.2003.12.4.26484.

[327] H.S. Debone, P.S. Lopes, P. Severino, C.M.P. Yoshida, E.B. Souto, C.F. da Silva, Chitosan/Copaiba oleoresin films for would dressing application, International Journal of Pharmaceutics, 555 (2019) 146-152 https://doi.org/10.1016/j.ijpharm.2018.11.054.

[328] N.M. Hromiš, V.L. Lazić, S.L. Markov, Ž.G. Vaštag, S.Z. Popović, D.Z. Šuput, N.R. Džinić, A.S. Velićanski, L.M. Popović, Optimization of chitosan biofilm properties by addition of caraway essential oil and beeswax, Journal of Food Engineering, 158 (2015) 86-93 https://doi.org/10.1016/j.jfoodeng.2015.01.001.

[329] S.H. Othman, S.A.M. Edwal, N.P. Risyon, R.K. Basha, R. A. Talib, Water sorption and water permeability properties of edible film made from potato peel waste, Food Science and Technology, 37 (2017) 63-70 https://doi.org/10.1590/1678-457x.30216.

[330] I. Corazzari, R. Nisticò, F. Turci, M.G. Faga, F. Franzoso, S. Tabasso, G. Magnacca, Advanced physico-chemical characterization of chitosan by means of TGA coupled on-line with FTIR and GCMS: Thermal degradation and water adsorption capacity, Polymer Degradation and Stability, 112 (2015) 1-9 https://doi.org/10.1016/j.polymdegradstab.2014.12.006.

[331] D. de Britto, S.P. Campana-Filho, Kinetics of the thermal degradation of chitosan, Thermochimica Acta, 465 (2007) 73-82 https://doi.org/10.1016/j.tca.2007.09.008.

[332] M. Kaya, S. Khadem, Y.S. Cakmak, M. Mujtaba, S. Ilk, L. Akyuz, Asier M. Salaberria, J. Labidi, A.H. Abdulqadir, E. Deligöz, Antioxidative and antimicrobial edible chitosan films blended with stem, leaf and seed extracts of Pistacia terebinthus for active food packaging, RSC Advances, 8 (2018) 3941-3950 https://doi.org/10.1039/c7ra12070b.

[333] J.F. Rubilar, R.M.S. Cruz, H.D. Silva, A.A. Vicente, I. Khmelinskii, M.C. Vieira, Physico-mechanical properties of chitosan films with carvacrol and grape seed extract, Journal of Food Engineering, 115 (2013) 466-474 https://doi.org/10.1016/j.jfoodeng.2012.07.009.

[334] J.T. Martins, M.A. Cerqueira, A.A. Vicente, Influence of α-tocopherol on physicochemical properties of chitosan-based films, Food Hydrocolloids, 27 (2012) 220-227 https://doi.org/10.1016/j.foodhyd.2011.06.011.

[335] M. Vargas, A. Albors, A. Chiralt, C. González-Martínez, Characterization of chitosan–oleic acid composite films, Food Hydrocolloids, 23 (2009) 536-547 https://doi.org/10.1016/j.foodhyd.2008.02.009.

[336] C. Edwards, R. Marks, Evaluation of biomechanical properties of human skin, Clinics in Dermatology, 13 (1995) 375-380 https://doi.org/10.1016/0738-081X(95)00078-T.

[337] F.H. Silver, L.M. Siperko, G.P. Seehra, Mechanobiology of force transduction in dermal tissue, Skin Research and Technology, 9 (2003) 3-23 https://doi.org/10.1034/j.1600-0846.2003.00358.x.

[338] C. Wiegand, U. Schönfelder, M. Abel, P. Ruth, M. Kaatz, U.C. Hipler, Protease and pro-inflammatory cytokine concentrations are elevated in chronic compared to acute wounds and can be modulated by collagen type I in vitro, Arch Dermatol Res, 302 (2010) 419-428 https://doi.org/10.1007/s00403-009-1011-1

[339] A. Ribeiro, T. Matamá, C.F. Cruz, A.C. Gomes, A.M. Cavaco-Paulo, Potential of human γD-crystallin for hair damage repair: insights into the mechanical properties and biocompatibility, International Journal of Cosmetic Science, 35 (2013) 458-466 https://doi.org/10.1111/ics.12065.

[340] T. Vanden Berghe, M. Kalai, G. Denecker, A. Meeus, X. Saelens, P. Vandenabeele, Necrosis is associated with IL-6 production but apoptosis is not, Cellular Signalling, 18 (2006) 328-335 https://doi.org/10.1016/j.cellsig.2005.05.003.

[341] K. Takei, A. Hashimoto-Hachiya, M. Takahara, G. Tsuji, T. Nakahara, M. Furue, Cynaropicrin attenuates UVB-induced oxidative stress via the AhR–Nrf2–Nqo1 pathway, Toxicology Letters, 234 (2015) 74-80 https://doi.org/10.1016/j.toxlet.2015.02.007.

[342] L.E. Tracy, R.A. Minasian, E.J. Caterson, Extracellular Matrix and Dermal Fibroblast Function in the Healing Wound, Advances in wound care, 5 (2016) 119-136 https://doi.org/10.1089/wound.2014.0561.

Annexes

Extraction processes for cynaropicrin present in leaves of *Cynara cardunculus* L. by, T. Bras, L. A. Neves, M. F. Ricardo, J. P. Crespo, (2019, April 10) EP3466936A1 [Online] Available: https://worldwide.espacenet.com/patent/search/family/059337729/publication/EP3466936A1?q=EP3466936A1

The present invention relates to methods for the extraction of the sesquiterpene lactone cynaropicrin, present in leaves of *Cynara cardunculus*. The invention contemplates the extraction of cynaropicrin, present in thistle leaves (*Cynara cardunculus*), where, before the extractive process, the biomass undergoes a size reduction in order to obtain a grain size of less than 400 µm. After this first step, the biological material in the present invention undergoes several types of independent extraction: solid-liquid batch, ultrasounds, microwaves or pressurized liquid solvent extraction. In the present invention, pure solvents, such as ethanol, ethyl acetate, as well as solvent mixtures, such as ethanol/water, are also presented as extraction solvents. The extraction process is controlled in relation to time, temperature, solid-liquid ratio and solvent variables for the set of processes shown, and depending on the extraction process, controlled in relation to stirring, ultrasonic power, microwave power and number of cycles of extraction.

These processes of cynaropicrin extraction have potential for application in the nutraceutical, pharmaceutical and chemical industries.

Description

“Extraction processes for cynaropicrin present in leaves of *Cynara cardunculus* L.”

Technical Field

The present application relates to methods for the extraction of the sesquiterpene lactone cynaropicrin, present in leaves of *Cynara cardunculus*. The extraction is carried out using four extraction methods, solid-liquid batch extraction, ultrasounds, microwaves, and extraction by pressurized liquid and pure solvents, such as ethanol and ethyl acetate, and a solvent mixture, such as ethanol/water mixture.

Background of the Invention

Belonging to the sesquiterpene lactone family, cynaropicrin is primarily recognized by the bitter taste it gives to food. Present in greater abundance in leaves of the family Asteraceae, it can be found in the commercially known artichoke (*Cynara cardunculus* var. Scolymus L.).

Based on its presence in *Cynara scolymus*, several previous studies have demonstrated its potential for application in the food, pharmaceutical and nutraceutical industries.

Originating from leaves or aerial parts of the *Cynara scolymus* plant, several studies have already been done and/or patented in order to demonstrate its potential for application, such as:

Assessment of ability to reduce cholesterol. For this, artichoke leaves were subjected to extraction techniques, confirming their potential for application in cholesterol reduction (Fritsche, Beindorff et al., 2002). The present invention is distinct from the cited study for using leaves of *Cynara cardunculus* and not commercially available artichoke products.

Study on the anti-hyperlipidemic potential of sesquiterpene lactones. With a study published in 2008, Shimoda et al. (Shimoda, Ninomiya et al., 2003) have demonstrated anti-hyperlipidemic potential, namely of cynaropicrin, from *Cynara scolymus* L., using a fractionated methanol extract to obtain the cynaropicrin-containing fraction, whereas the present invention does not use additional extraction steps, obtaining a fraction rich in cynaropicrin with a single extraction step.

Determination of antispasmodic activity. Emendörfer et al. (Emendörfer, Emendörfer et al., 2005) have demonstrated antispasmodic activity of extract fractions, and in particular of cynaropicrin, from *Cynara scolymus*, distinct from the present invention by the use of extract fractionation and a long extraction time (7 days), therefore becoming a time consuming and expensive process, as opposed to the present invention.

Despite a high correlation between the descriptions of the biological potential of cynaropicrin and the extracts derived from the *Cynara scolymus* plant, also cynaropicrin derived from other plants, or even the synthetic form, has been previously studied, showing its application potential, namely in response to anti-inflammatory processes (Cho, Baik et al., 2000), and its cytotoxic and pro-apoptotic activity in cancer cells (Cho, Kim et al., 2004), with very positive and satisfactory results in both settings. Present in other species of the family Astearecae, the extraction of cynaropicrin has been studied based on different extraction techniques. In 2013, a chemical characterization study of the *Cynara cardunculus* var. Altilis plant was published, where for the first time cynaropicrin was quantified in this *Cynara cardunculus* variety (Ramos, Guerra et al. 2013). This invention has used a long extraction time of 7 h and the use of environmentally aggressive solvents. The present invention can be distinguished by the use of environmentally friendly solvents, reduced extraction times and yield of higher cynaropicrin concentrations (40 mg/g dry weight versus 55 mg/g dry weight). More recently, the same authors have demonstrated the potential of said sesquiterpene lactone in reducing cell viability of breast cancer cells with a triple-negative phenotype (Ramos, Guerra et al., 2014).

Despite the well-known biological potential of cynaropicrin, no processes have been developed to obtain higher concentrations of cynaropicrin from leaves of the *Cynara cardunculus* plant.

In order to understand and expand the potential of cynaropicrin application, either in terms of biological potential, and consequently therapeutic, or for food applications, the extraction methods previously published by several authors aimed only at obtaining extract fractions (S) containing cynaropicrin, not having the maximization of the cynaropicrin extraction as a central goal. The following currently published extraction methods can be found:

In order to study the healing properties of *Cynara scolymus* L. extracts, extraction was carried out using ethanol solutions in solid-liquid batch mode, in a proportion of 45% ethanol, obtaining a composition with 1% of cynaropicrin (Ishida, Kojima et al., 2010), as opposed to extracts obtained with the present invention, where pure solvents or an ethanol/water mixture at 40% ethanol are used, obtaining an average of 27% of cynaropicrin depending on the selected extraction method.

In order to evaluate its potential in cholesterol reduction, artichoke leaves were submitted to solid-liquid batch extraction processes, also known as maceration, and purification of their bioactive compounds, namely cynaropicrin, using purification and isolation processes by liquid chromatography (Sephadex) (Fritsche, Beindorff et al., 2002), where isolation is not necessary in the present invention to obtain an extract rich in cynaropicrin.

In the described art, in EP 2379092B1, aerial parts of *Cynara scolymus* were subjected to extraction using a heating mantle percolator, with an extraction temperature of 70 °C, and with 70% ethanol as an extraction solvent, obtaining at the end of the process 10% to 13% of cynaropicrin (Bombardelli 2013). In addition to obtaining higher concentrations of cynaropicrin, lower temperatures (between 30 and 60 °C) are used in the present invention.

More recently, capitula of *Cynara scolymus* plant were also subjected to extraction techniques with ethanol/water mixtures in a 7:3 ratio v/v, having in its final composition 1% of cynaropicrin (Bombardelli, Fontana et al. 2008), thereby obtaining significantly lower values as compared to the values obtained using the methods described in the present patent application.

In 2013 a characterization study of the *Cynara cardunculus* var. Altilis plant was published, where leaf extraction was carried out using Soxhlet extraction, with an extraction time of 7 h having cynaropicrin quantified for the first time in this variety of the *Cynara cardunculus* plant (Ramos, Guerra et al., 2013). The proposed invention differs from the invention of Ramos et al. in the use of extraction methods with an extraction time of less than 7 h and in obtaining higher percentages of cynaropicrin.

However, for the extraction of the sesquiterpene lactone cynaropicrin, only maceration (solid-liquid batch) stands out, with solvents such as methanol or ethanol/water mixture, being mostly followed by purification techniques in order to separate the desired fractions.

The present patent application discloses new methods of extracting the sesquiterpene lactone cynaropicrin, present in the leaves of the *Cynara cardunculus* L. plant, such as solid-liquid batch, ultrasounds, microwaves and solvent by pressurized liquid, using different extraction solvents, ethanol, ethanol/water and ethyl acetate. With the methodology described in this patent application for extraction of cynaropicrin, a yield increase and a reduction in the extraction time are achieved as compared to the methodology of the prior art.

Summary of the Invention

The present patent application describes novel methods for the extraction of the sesquiterpene lactone cynaropicrin present in the leaves of *Cynara cardunculus*.

In one embodiment the method of extracting cynaropicrin from leaves of *Cynara cardunculus* comprises the following steps:

- lyophilisation and grinding of *Cynara cardunculus* leaves, with a particle size of less than 400 µm;
- selecting a solvent in its pure state or in a mixture;
- extraction of the lyophilised biomass with the solvent selected in the previous step in a closed container protected from light.

In another embodiment, the solvent used in the method for extraction of cynaropicrin is ethanol, ethyl acetate or an ethanol/water mixture.

In one embodiment, the container is subjected to the action of bath ultrasound equipment or with a probe during the extraction step. In another embodiment, the extraction step has a duration of between 15 and 240 min, takes place at a temperature between 25 and 60 °C and at an ultrasonic power between 5 and 100 kHz.

In one embodiment, the container is subjected to the action of stirring equipment during the extraction step. In another embodiment, the extraction step has a duration of between 15 and 240 min, takes place at a temperature between 25 and 60 °C and at a stirring between 300 and 900 rotations per minute.

In one embodiment, the container is subjected to the action of ultrasound equipment during the extraction step. In another embodiment, the extraction step has a duration of between 5 and 120 min, takes place at a temperature between 25 and 80 °C and at a microwave power between 500 and 2000 W.

In one embodiment, the container is subjected to the action of pressurized liquid extraction equipment during the extraction step. In another embodiment, the extraction step takes place at a temperature between 25 and 150 °C and over 1 to 5 extraction cycles.

In one embodiment, the biomass/solvent ratio is between 1/4 and 1/100.

In another embodiment, the ethanol/water ratio is 40/60.

Detailed Description of the Invention

The present patent application relates to novel methods for the extraction of the sesquiterpene lactone cynaropicrin, present in leaves of *Cynara cardunculus*. The extraction is carried out using four methods of extraction, solid-liquid batch extraction, ultrasounds, microwaves and extraction by

pressurized liquid and pure solvents, such as ethanol and ethyl acetate, and a solvent mixture, such as an ethanol/water mixture. After the extraction process, no additional extraction and/or purification steps are required.

Collection and storage

Leaves of *Cynara cardunculus* are harvested fresh before the summer high temperatures in order to avoid the presence of dry biomass. After collecting fresh material, it is immediately lyophilised in any commercially available lyophiliser for the removal of water for 5 to 7 days and/or until a constant mass value is obtained, indicative of complete removal of the moisture present in the fresh leaves. After obtaining a constant mass value, the material is grounded in a knife mill equal to those commercially available, with a grain size of less than 400 µm. After grinding, the material is vacuum packed (optional) in order to avoid subsequent water absorption. This set of processes, from collection to storage, allows for the material to be stored at room temperature, with a reduced storage volume and without deterioration of its compounds of interest.

Extraction Methods

The following methodology is used for extraction of the sesquiterpene lactone cynaropicrin.

Ultrasonic extraction

For ultrasonic extraction, the following pure solvents or solvent mixtures can be used as extraction solvents: ethanol, ethyl acetate, and ethanol/water. The extraction procedure aims at placing together, in a closed container protected from light, subjected to ultrasound, bath, probe or similar equipment, the lyophilised biomass and a solvent or mixture of solvents, at a particular biomass/solvent ratio, during a particular period of time, at a particular temperature and at a particular ultrasonic power value. For this procedure, any ultrasonic wave emission device can be used, from probes to baths, with no need for specification. In the present invention, the extraction time is between 15 min and 4 h, preferably between 20 and 90 min, the extraction temperature is between 25 °C and 60 °C, preferably between 40 and 60 °C, and the ultrasonic power value is between 5 and 100 kHz, preferably between 10 and 30 kHz. As a biomass/solvent ratio, it has a ratio between 1/4 and 1/100, preferably between 1/10 and 1/50. After extraction, the supernatant is quantified by high-pressure liquid chromatography (HPLC) and results are expressed as mg compound/g lyophilised weight and % of cynaropicrin in the extract.

Solid-liquid batch extraction

For solid-liquid batch extraction, the following pure solvents or solvent mixtures can be used as extraction solvents: ethanol, ethyl acetate, ethanol/water. The extraction procedure is aimed at placing together, in a closed container and protected from light, with stirring and heating, the lyophilised biomass and the solvent or mixture of solvents, at a particular biomass/solvent ratio, for a particular period of time, at a particular temperature and at a particular stirring rate. For this procedure, any heating and stirring device can be used, with no need for specification. In the present invention, the extraction time is between 20 min and 4 h, preferably between 30 and 90

min, the extraction temperature is between 25 °C and 60 °C, preferably between 30 and 50 °C and the stirring value is between 300 and 900, preferably between 400 and 700 rotations per minute. As a biomass/solvent ratio, it has a ratio between 1/4 and 1/100, with 1/10 and 1/50 being preferred. After extraction, the supernatant is quantified by high-pressure liquid chromatography (HPLC) and results are expressed as mg compound/g lyophilised weight and % of cynaropicrin in the extract.

Microwave extraction

For microwave extraction, the following pure solvents or solvent mixtures can be used as extraction solvents: ethanol, ethyl acetate, and ethanol/water. The extraction procedure is aimed at placing together in a closed container and protected from light, subject to suitable microwave equipment, the lyophilised biomass and the solvent or solvent mixture at a particular biomass/solvent ratio, for a particular period of time, at a particular temperature and at a particular microwave power value. For this procedure, any commercially available microwave digestion device can be used, with no need for specification. In the present invention, the extraction time is between 5 min and 2 h, preferably between 15 and 60 min, the extraction temperature is between 25 °C and 80 °C, preferably between 30 and 65 °C, and the value of microwave power is between 500 and 2000 W, preferably between 700 and 1500 W. As a biomass/solvent ratio, it has a ratio between 1/4 and 1/100, with 1/10 and 1/50 being preferred. After extraction, the supernatant is quantified by high-pressure liquid chromatography (HPLC) and results are expressed as mg compound/g lyophilised weight and % of cynaropicrin in the extract.

Pressurized liquid solvent extraction

For pressurized liquid solvent extraction, the following pure solvents and solvent mixtures can be used as extraction solvents: ethanol, ethyl acetate, and ethanol/water. The extraction procedure is aimed at placing together, in a closed container protected from light, subject to proper equipment for pressurized liquid solvent extraction, the lyophilised biomass and the solvent or solvent mixture at a particular biomass/solvent ratio, at a particular temperature and with a particular number of extraction cycles. For this procedure, any commercially available pressurized liquid solvent extraction equipment can be used, with no need for specification. In the present invention, an extraction temperature is presented, a temperature between 25 and 150 °C, preferably between 30 and 90 °C, and extraction cycles between 1 and 5. As a biomass/solvent ratio, it has a ratio between 1/4 and 1/100, preferably between 1/10 and 1/60. After extraction, the supernatant is quantified by high-pressure liquid chromatography (HPLC) and results are expressed as mg compound/kg lyophilised weight and % of cynaropicrin in the extract.

Examples

Ultrasonic extraction:

Ultrasonic extraction with a solid-liquid ratio of 1/16, using ethyl acetate as an extraction solvent, an extraction temperature of 40 °C, an ultrasonic power of 42 kHz, and an extraction time of 60 min. The results obtained have shown the attainment of an extract with a concentration of 46 mg of cynaropicrin/g of lyophilisate, 38% in extract base.

Pressurized liquid solvent extraction:

Pressurized liquid solvent extraction with a solid-liquid ratio of 1/16, using ethanol as an extraction solvent, an extraction temperature of 40 °C and two extraction cycles. The results obtained have shown the attainment of an extract with a concentration of 50 mg of cynaropicrin/g of lyophilisate, and 21% of cynaropicrin in the extract.

Solid-liquid batch extraction:

Solid-liquid batch extraction with a solid-liquid ratio of 1/16, using ethanol as an extraction solvent, an extraction temperature of 40 °C, stirring at 700 rotations per minute and an extraction time of 60 min. The results obtained have shown the attainment of an extract with a concentration of 57 mg of cynaropicrin/g of lyophilisate weight, and 27% of cynaropicrin in the extract.

Microwave extraction:

Microwave extraction with a solid-liquid ratio of 1/16, using the ethanol/water mixture as an extraction solvent, an extraction temperature of 40 °C, a microwave power of 1000 W and an extraction time of 15 min. The results obtained have shown the attainment of an extract with a concentration of 18,51 mg of cynaropicrin/g of lyophilisate, and 11% of cynaropicrin in the extract.

With the methods and solvents disclosed in this patent application, novel methods of extracting cynaropicrin present in the leaves of *Cynara cardunculus* are achieved.

With the methods and solvents disclosed herein, it is possible to extract sesquiterpene lactone from all varieties of *Cynara cardunculus*.

With the present method of solid-liquid batch extraction or maceration, higher values of cynaropicrin are obtained as compared to those obtained in other prior art methods, using different pure solvents and different ethanol/water ratios, as well as lower temperatures, which leads to lower energy costs.

It is important to note that, unlike what happens in the present methodology, ethyl acetate is not normally used for the first extraction phase and is always used as a purification solvent in later phases. With the methodology disclosed in this patent application, it is possible to obtain higher percent values of cynaropicrin than previously published.

The present description is, naturally, not in any way restricted to the embodiments presented herein and a person of ordinary skill in the art may foresee many possibilities of modifying it without departing from the general idea as defined in the claims. The preferred embodiments described above are obviously combinable with each other. The following claims further define preferred embodiments.

References

Bombardelli, E. (2013). "Compositions comprising a lipophilic extract of Zingiber officinale and an extract of *Cynara scolymus*", Google Patents.

Bombardelli, E., et al. (2008). "*Cynara scolymus* extracts and compositions containing them", Google Patents.

Cho, J. Y., et al. (2000). "In vitro anti-inflammatory effects of cynaropicrin, a sesquiterpene lactone, from Saussurea lappa" European journal of pharmacology 398(3): 399-407.

Cho, J. Y., et al. (2004). "Cytotoxic and pro-apoptotic activities of cynaropicrin, a sesquiterpene lactone, on the viability of leukocyte cancer cell lines" European journal of pharmacology 492(2): 85-94.

Emendörfer, F., et al. (2005). "Antispasmodic activity of fractions and cynaropicrin from *Cynara scolymus* on guinea-pig ileum" Biological and Pharmaceutical Bulletin 28(5): 902-904.

Fritsche, J., et al. (2002). "Isolation, characterization and determination of minor artichoke (*Cynara scolymus* L.) leaf extract compounds" European Food Research and Technology 215(2): 149-157.

Ishida, K., et al. (2010). "Effects of artichoke leaf extract on gastric mucosal injury in rats" Biological and Pharmaceutical Bulletin 33(2): 223-229.

Ramos, P., et al. (2014). "Antitumoral and antioxidant activities of lipophilic and phenolic extracts from *Cynara cardunculus* L. var. Altilis (DC)" Planta Medica 80(16): P1L16.

Ramos, P.A., et al. (2013). "Lipophilic extracts of *Cynara cardunculus* L. var. Altilis (DC): a source of valuable bioactive terpenic compounds" Journal of agricultural and food chemistry 61(35): 8420-8429.

Shimoda, H., et al. (2003). "Anti-hyperlipidemic sesquiterpenes and new sesquiterpene glycosides from the leaves of artichoke (*Cynara scolymus* L.): structure requirement and mode of action" Bioorganic & medicinal chemistry letters 13(2): 223-228.

CLAIMS

1 A method for extracting cynaropicrin from leaves of *Cynara cardunculus*, characterized in that it comprises the following steps:

- lyophilisation and grinding of leaves of *Cynara cardunculus*, with a particle size of less than 400 µm;

- selecting a solvent in its pure state or in a mixture;

- extraction of the lyophilised biomass with the solvent selected in the previous step in a closed container and protected from light.

2 A method for extracting cynaropicrin according to claim 1, wherein the solvent is ethanol, ethyl acetate or an ethanol/water mixture.

3 A method for extracting cynaropicrin according to any one of claims 1 or 2, wherein in the extraction step the container is subjected to the action of a bath or probe ultrasound equipment.

4 A method for extracting cynaropicrin according to any claim 3, wherein the extraction step has a duration of between 15 and 240 min, takes place at a temperature between 25 and 60 °C and at an ultrasonic power between 5 and 100 kHz.

5 A method for extracting cynaropicrin according to any one of claims 1 or 2, wherein in the extraction step the container is subjected to the action of a stirring equipment.

6 A method for extracting cynaropicrin according to claim 5, wherein the extraction step has a duration of between 15 and 240 min, takes place at a temperature between 25 and 60 °C and with a stirring of between 300 and 900 rotations per minute.

7 A method for extracting cynaropicrin according to any one of claims 1 or 2, wherein in the extraction step the container is subjected to the action of a microwave equipment.

8 A method for extracting cynaropicrin according to claim 7, wherein the extraction step has a duration of between 5 and 120 min, takes place at a temperature between 25 and 80 °C and at a microwave power of between 500 and 2000 W.

9 A method for extracting cynaropicrin according to any one of claims 1 or 2, wherein in the extraction step the container is subjected to the action of a pressurized liquid extraction equipment.

10 A method for extracting cynaropicrin according to claim 9, wherein the extraction step takes place at a temperature between 25 and 150 °C and over 1 to 5 extraction cycles.

11 A method for extracting cynaropicrin according to any one of claims 1-10, wherein the biomass/solvent ratio is between 1/4 and 1/100.

12 A method of extracting cynaropicrin according to any one of claims 2-11, wherein the ethanol/water ratio is 40/60.