Universidade de Aveiro Departamento de Química
Ano 2020

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Ciência e Tecnologia Alimentar e Nutrição, realizada sob a orientação científica do Doutor Jorge M. A. Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro, e da Doutora Maria Manuela E. Pintado, Professora Associada da Escola Superior de Biotecnologia da Universidade Católica Portuguesa, Porto.

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Aos meus pais, com amor.

“*What’s your motivation? Mom’s smile and Dad’s pride.*”

o júri

presidente

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palavras-chave

Extração assistida por alta pressão, análise por superfície de resposta, otimização do processo de extração, compostos bioativos, compostos individuais, atividades biológicas, citotoxicidade, genotoxicidade, urtiga, segurelha, sumo de cenoura

resumo

Tanto a urtiga como a segurelha são ervas perenes geralmente encontradas na região do Mediterrâneo, sendo tradicionalmente usadas como ervas medicinais devido às propriedades biológicas associadas à sua composição química. A primeira parte deste trabalho teve como objetivo avaliar o efeito da extração assistida por alta pressão (EAP) e sua otimização pela metodologia de superfície de resposta, utilizando um planeamento de composto central de face centrada, de modo a obter extratos com elevado conteúdo em compostos bioativos. Foram avaliados: o efeito do nível de pressão, o tempo de extração e a concentração de solvente (etanol:água), bem como o impacto da EAP sobre a concentração de fenólicos totais (FT), flavonóides, pigmentos e atividade antioxidante. Os resultados demonstraram que os dados experimentais poderiam ser bem ajustados a modelos matemáticos de segunda ordem. Para o processo de extração usando folhas de urtiga, as condições ideais para maximização do rendimento, FT e atividade antioxidante foram 200 MPa, 10,2-15,6 min e 0% etanol (extratos aquosos); enquanto que para as folhas de segurelha, os modelos previram condições ideais de extração que variam entre 200 e 500 MPa, tempo de extração de 1-20 minutos e concentração de etanol de 0-70%. Em comparação com a extração à pressão atmosférica, EAP permitiu aumentar o rendimento em cerca de 50,5%; FT cerca de 84,4%; e atividade antioxidante em cerca de 77,7% para extratos de urtiga; e um aumento de cerca de 40% para todos os compostos, 29, 48 e 70% para atividade antioxidante pelos ensaios FRAP, DPPH e ABTS, respetivamente, para os extratos de segurelha.

Na segunda parte deste trabalho, foram estudadas as propriedades biológicas e a toxicidade dos extratos otimizados. Cada extrato foi caracterizado no que concerne ao seu perfil de compostos fenólicos individuais e diferentes propriedades biológicas, como atividade antioxidante, atividade pró-oxidante (capacidade de degradação de DNA), e também atividade antimicrobiana, anti-biofilme e atividade anti-hipertensiva. A citotoxicidade dos extratos e seu efeito anti-proliferativo em linhas celulares tumorais humanas também foram avaliados. Para a urtiga, os extratos obtidos a 200 MPa, 10 min, 35 e 70% de etanol foram os que apresentaram maiores concentrações de ácidos fenólicos e flavonóides, tais como o ácido clorogénico, ácido isoferúlico e rutina; sendo também os que apresentam melhores resultados em todas as atividades biológicas estudadas; enquanto que para a segurelha, o extrato obtido a 348 MPa, 20 min, 35% de etanol, mostrou-se o mais interessante, pois apresentou uma elevada concentração de compostos fenólicos individuais, como o ácido rosmarínico e ácido salvianólico A. Os extratos obtidos por EAP apresentaram potencial como antioxidantes e protetores de DNA, uma vez que foram capazes, não apenas de causar menores danos na molécula de DNA do que os controlos, como também foram capazes de a proteger contra danos causados devido ao stress oxidativo. Em relação à citotoxicidade, observou-se que os extratos de EAP, numa concentração de 0,5 e 1,0 mg/mL (para segurelha e urtiga, respectivamente), não foram prejudiciais ao metabolismo da linhagem celular HT29; enquanto que os controlos em concentrações superiores (>1.0 mg/mL) causaram uma ligeira redução da atividade metabólica. Por fim, todos os extratos causaram uma inibição da viabilidade de três linhagens celulares cancerígenas (>2.0 mg/mL para Caco-2, HeLa, e TR146), indicando que estes extratos poderão apresentar uma potencial atividade anti-tumoral.

A última parte deste trabalho teve como objetivo avaliar o efeito da suplementação de sumo de cenoura com extrato de segurelha nas características finais do sumo, após armazenamento durante 15 dias em refrigeração. Os sumos suplementados apresentaram contagens microbianas mais baixas do que os não suplementados e, geralmente, não apresentaram alterações significativas ($p>0,05$) no pH nem na cor. Em relação aos fenólicos totais e flavonóides, bem como relativamente à atividade antioxidante, os valores foram geralmente mais altos para os sumos suplementados, o que foi comprovado pela elevada correlação encontrada entre a concentração de fenólicos totais e a atividade antioxidante determinada pelos métodos de ABTS e FRAP.

keywords

High pressure assisted extraction, response surface methodology, extraction process optimization, bioactive compounds, individual compounds, biological activities, cytotoxicity, genotoxicity, stinging nettle, winter savory, carrot juice

abstract

Both stinging nettle and winter savory are both perennial herbs usually found in the Mediterranean area, being traditionally used as medicinal herbs against several ailments due to the biological properties associated to their rich chemical composition. The first part of the present work aimed to evaluate the effect of high pressure assisted extraction (HPE) and its optimization by response surface methodology using a central composite face centered design in order to obtain extracts with high content of bioactive compounds and improved bioactivities. The effect of pressure level, extraction time, and solvent (ethanol:water) concentration was evaluated, as also the impact of HPE on total phenolics (TPC), flavonoids, pigments, and antioxidant activity. Results showed that experimental data could be well fitted to second-order polynomial mathematical models. For stinging nettle leaves extraction process, the optimal conditions for maximization of extraction yield, TPC, and antioxidant activity were 200 MPa, 10.2-15.6 min, and 0% ethanol (aqueous extracts); while for winter savory leaves, the models predicted optimal conditions ranging from 200-500 MPa, extraction time 1-20 min, and ethanol concentration 0-70%. In comparison with control extraction at atmospheric pressure, HPE allowed to increase the extraction yield about 50.5%; TPC about 84.4%; and antioxidant activity about 77.7% for stinging nettle extracts; and an increase of about 40% for all compounds, 29, 48, and 70% for antioxidant activity by FRAP, DPPH and ABTS assays, respectively, for winter savory extracts.

In the second part of this work, the biological properties and toxicity of the controls and the optimized extracts from both herbs were studied. The studied extracts were obtained at pressure levels ranging from 200 and 500 MPa, 10-20 minutes, 0-70% ethanol. Each extract was characterized for their individual compounds profile and different biological properties, such as antioxidant activity, pro-oxidant activity (DNA degradation capacity), as also its antimicrobial, antibiofilm, and antihypertensive activities. The extracts cytotoxicity and antiproliferative effect on human tumour cell lines were also evaluated. For stinging nettle, the extracts obtained at 200 MPa, 10 min, 35 and 70% ethanol were the ones presenting higher concentrations of TPC and flavonoids, such as chlorogenic acid, isoferulic acid, and rutin, being also the ones with better results concerning all the studied biological activities; while for winter savory, the extract obtained at 348 MPa, 20 min, 35% ethanol showed to be the most interesting, since it presented a high concentration of individual phenolic compounds, such as tuberonic acid glucoside, rosmarinic acid, salvianolic acid A. HPE extracts showed potential antioxidant and DNA protective activities, since they were able not only to cause fewer damages in the DNA molecule than the controls, but also were able to protect it against the damage caused by oxidative stress. Concerning the cytotoxicity, it was observed that HPE extracts, in a concentration of 0.5 and 1.0 mg/mL, were not harmful to HT29 cell lines, but the control extracts at higher concentration (>1.0 mg/mL) showed that the extracts can slightly reduce the metabolic activity. Finally, all the extracts showed to inhibit the viability of three cancerous cell lines (>2.0 mg/mL for Caco-2, HeLa, and TR146), indicating that these extracts may present a potential antitumoral activity.

The last part of this work aimed to evaluate the effect of supplementation of carrot juice with winter savory leaves extract, on the final juice characteristics, after storage for 15 days under refrigeration. Supplemented juices presented lower microbial counts than the non-supplemented ones, and, generally, did not present significant changes ($p>0.05$) in pH or colour. Concerning the total phenolics and total flavonoids, as well as antioxidant activity, the values were generally higher in supplemented juices, which was proven by the high correlation found between total phenolics content and antioxidant activity by ABTS and FRAP assays.

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LIST OF ABBREVIATIONS

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

AChE – acetylcholinesterase

ANOVA – analysis of variance

BHA – butylated hydroxyanisole

CAE – caffeic acid equivalents

CCD – central composite face-centred design

Chl total – total chlorophyll

ChlA – chlorophyll a

ChlB – chlorophyll b

CUPRAC – cupric reducing antioxidant capacity

DM – dry matter

DNA – deoxyribonucleic acid

DOE – design of experiments

DPPH – 2,2-diphenyl-1-picrylhydrazyl

DW – dry weight

EC_{50} – half maximal effective concentration

ESI-MS – electrospray ionization-mass spectrometry

EtOH – ethanol

FRAP – ferric reducing antioxidant power

FW – fresh weight

GAE – gallic acid equivalents

GC-MS – gas chromatography–mass spectrometry

HCl – hydrochloric acid

HD – hydrodistillation

HPE – high pressure assisted extraction

HPLC – high-performance liquid chromatography

HPLC-DAD/ESI-MS – high-performance liquid chromatography with diode array detector coupled to mass spectrometry using the electrospray ionization interface

HPP – high pressure processing

IC_{50} – half maximal inhibitory concentration

LC-DAD – liquid chromatography with diode array detector

MBC – minimum bactericidal concentration

MIC – minimum inhibitory concentration

MLC – minimum lethal concentration

MLS – method of least square

MPa – megapascal

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MW – microwaves

MWE – microwaves assisted extraction

NF-κB – nuclear factor kappa B

NMR – nuclear magnetic resonance

ORAC – oxygen radical absorbance capacity

PEF – pulsed electric fields

PEFE – pulsed electric fields assisted extraction

RP-HPLC – reverse-phase high-performance liquid chromatography

RSD – relative standard deviation

RSM – response surface methodology

RT – room temperature

SC-CO_2 – supercritical fluid extraction using carbon dioxide

SEM – scanning electron microscope

SFE – supercritical fluid extraction

TEM – transmission electron microscope

TFC – total flavonoids content

TPC – total phenolic compounds

TROLOX – 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

UAE – ultrasounds assisted extraction

US – ultrasounds

UV/Vis – ultraviolet/visible spectroscopy

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LIST OF PUBLICATIONS

The current thesis is based on the following publications:

Published papers in peer reviewed journal (3):

- **Moreira, S.A.,** Silva, S., Costa, E., Saraiva, J.A., Pintado, M. (2020) - Effect of high hydrostatic pressure extraction on biological activities from stinging nettle extracts. Food and Function. doi: 10.1039/c9fo02442e **(Article 4)**
- **Moreira, S.A.**, Alexandre, E.C., Pintado, M., Saraiva, J.A. (2019) - Effect of emergent non-thermal extraction technologies on bioactive individual compounds profile from different plant materials. Food Research International. 115: 177-190. **(Article 1)**
- Alexandre, E.C., Castro, L.G., **Moreira, S.A.**, Pintado, M., Saraiva, J.A. (2017) - Comparison of emerging technologies to extract high-added value compounds from fruit residues: pressure- and electro-based technologies. Food Engineering Reviews. 9:3. 190-212.
- Alexandre, E.C., **Moreira, S.A.**, Castro, L.G., Pintado, M., Saraiva, J.A. (2018) - Emerging technologies to extract high added value compounds from fruit residues: Sub/supercritical, ultrasound-, and enzyme-assisted extractions. Food Reviews International. 34:6. 581-612.

Submitted papers in peer reviewed journals (5):

- **Moreira, S.A.**, Pintado, M., Saraiva, J.A. - Optimization of high hydrostatic pressure assisted extraction of stinging nettle leaves using response surface methodology experimental design. **(Article 2)**
- **Moreira, S.A.**, Pintado, M., Saraiva, J.A. - Optimization of antioxidant activity and bioactive compounds extraction of winter savory leaves by high hydrostatic pressure. **(Article 3)**
- **Moreira, S.A.**, Silva, S., Costa, E., Pinto, S., Sarmento, B., Saraiva, J.A., Pintado, M. - Effect of high hydrostatic pressure extraction on biological activities and phenolics composition of winter savory leaves extracts. **(Article 5)**
- **Moreira, S.A.**, Pintado, M., Saraiva, J.A. - Effect of winter savory leaves extract obtained by high hydrostatic pressure on quality of carrot juice. **(Article 6)**

Book chapters (1):

- Alexandre, E.C., **Moreira, S.A.**, Pintado, M., Saraiva, J.A. (2017) - Emergent extraction technologies to valorize fruit and vegetable residues, in Agricultural Research Updates, Vol. 17, Nova Science Publishers, Editors P. Gorawala and S. Mandhatri

Oral communications (3):

- **Moreira, S.A.**, Pintado, M., Saraiva, J.A. Research Summit, 2019, Universidade de Aveiro, Aveiro, Portugal: "Evaluation of high-pressure as a novel non-thermal extraction technology to obtain extracts with improved biological activities from medicinal plants"
- **Moreira, S.A.**, Pintado, M., Saraiva, J.A. IFT18 - A Matter of Science + Food, 2018, in Chicago, Illinois, USA: "High pressure as a new non-thermal extraction methodology to obtain extracts with improved biological activities. A case study on stinging nettle"
- **Moreira, S.A.**, Pintado, M., Saraiva, J.A. XII Jornadas da Etnobotânica, 2017, in Fornos de Algodres, Portugal: "Estudo da Alta Pressão como uma nova técnica para extração não-térmica de extratos com atividades biológicas melhoradas. Caso de estudo: Urtiga"

Posters (4):

- **Moreira, S.A.**, Silva, S., Veiga, M., Costa, E., Pintado, M., Saraiva, J.A. XII CIBIA, 2019, in Faro, Portugal: "Optimization of high hydrostatic pressure assisted extraction of stinging nettle leaves using an RSM experimental design"
- **Moreira, S.A.**, Silva, S., Costa, E., Saraiva, J.A., Pintado, M. XIV EQA, 2018, in Viana do Castelo, Portugal: «Effect of high hydrostatic pressure extraction on the genotoxicity and cytotoxicity of herbal extracts: a case study on stinging nettle"
- **Moreira, S.A.**, Pintado, M., Saraiva, J.A. 56th EHPRG, 2018, in Aveiro, Portugal: «Effect of high hydrostatic pressure extraction on biological activities of stinging nettle extracts»
- **Moreira, S.A.**, Alexandre, E.M.C., Pintado, M., Saraiva, J.A. 55th EHPRG, 2017, in Poznan, Poland: «Optimization of high hydrostatic pressure extraction by response surface methodology of bioactive compounds from stinging nettle»

Other publications (out of this thesis context):

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- Fernandes, P.R. et al. (2019) - Hyperbaric storage at variable room temperature – a new preservation methodology for minced meat compared to refrigeration. Journal of the Science of Food and Agriculture. 99:7. 3276-3282
- Pinto, C. et al. (2017) - Impact of different hyperbaric storage conditions on microbial, physicochemical and enzymatic parameters of watermelon juice. Food Research International. 99: 123-132
- Duarte, R.V. et al. (2017) - Whey cheese longer shelf-life achievement at variable uncontrolled room temperature and comparison to refrigeration. Journal of Food Processing and Preservation. 41:6. e13307
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Book Chapters (3):

- Alexandre, E.M.C. et al (2019) - Non-thermal Food Processing/Preservation Technologies, in Saving Food: Production, Supply Chain, Food Waste and Food Consumption, Editor: Elsevier- Academic Press, Editor Charis M. Galanakis
- Koubaa, M. et al (2017) - Pulsed Electric Field Processing of Fruit Juices, in Fruit Juices, Ed.1, Chapter 22, Publisher: Academic Press, Editors G. Rajauria and B. Tiwari, pp.437-449
- Santos, M.D. et al (2016) - Interaction of compounds as affected by different emerging processing techniques, in Nutraceutical and Functional Food Components: Effects of Innovative Processing Techniques, 1st ed., Elsevier, Editor Charis M. Galanakis

SYNOPSIS

This thesis is divided in seven distinct chapters.

Chapter I comprises the state of the art concerning (i) the importance of bioactive compounds (ii) the state of art of the two herbs under study; (iii) high-pressure as an extraction and food processing methodology; (iv) response surface methodology and experimental design.

Chapter II provides de objectives and the workplan.

Chapter III presents a detailed description of the materials and methods used in this work.

Chapter IV presents and discusses the results regarding the optimization of the extraction process for both herbs.

Chapter V presents and discusses the results regarding the biological activities of each extract for both herbs.

Chapter VI provides the results obtained for the case study: fortification of a beverage with one of the extracts.

Lastly, Chapter VII provides the final considerations and main conclusions of this thesis, as well as several suggestions for future work.

CHAPTER I

GENERAL INTRODUCTION

Partially adapted from:

Moreira, S.A. et al. (2019) - Effect of emergent non-thermal extraction technologies on bioactive individual compounds profile from different plant materials. Food Research International 115: 177-190.

Summary

1. Contextualization

Natural products and its derivatives represent more than 50% of all the worldwide used drugs, being about 25% of all drugs formulation based on herbal compounds and its derivatives. Most population still uses herbal medicine on its daily needs, and according to WHO, 80% of world population accepts and uses plant derived drugs for treatments and disease prevention **(Gurib-Fakim, 2006)**. Pharmacognosy was defined, for the first time, by Seidler, in 1815, as the science which has the task to learn everything about drugs originating from plants or animals in all aspects **(Gurib-Fakim, 2006)**. It is possible to draw three general aspects relatively to herbal extracts development: (i) therapeutically effective herbal extracts do not contain just one active constituent or a few structurally similar compounds, but several often structurally unrelated groups of compounds may occur with activity that influences the same pathological situation; (ii) the bioavailability of certain active constituents in a herb can be improved by accompanying compounds (co-effectors) present in the same product; and (iii) the manufacturing process can eliminate or degrade unwanted active constituents in order to produce safe extracts with retained therapeutic efficacy **(Nahrstedt and Butterweck, 2010)**. Since ancient times, several herbs have been used in folk medicine due to the properties of their components; nowadays, these biological activities have been proven and new discoveries of their potential are constantly reported for the pharmaceutical and food industries, for their use as new drugs or for food preservation **(Grosso et al., 2009b)**. Herbal extracts are very attractive not only in the modern phytotherapy but also in the food industry, as spices and additives.

Extraction is the first step for obtaining important bioactive compounds present in herbal materials, and it can be described as a mass transport phenomenon where components present in a matrix are transferred into a solvent **(Lee et al., 2011)**. The growing interest about bioactive compounds from fruits and vegetables is related to the consumer preference of natural additives over synthetic ones **(Prasad et al., 2009d)**. In general, bioactive compounds, such as phenolic compounds, are secondary metabolites of plants, being present in much lower levels than constitutive molecules (lipids, proteins, and carbohydrates). The major problem is that those bioactive compounds, such as flavonoids, anthocyanins, etc., are present in insoluble structures (for instance, vacuoles of plant cells or bilayers of lipoproteins), which may turn its extraction into a challenge **(Corrales et al.,**

2008). Many organic compounds in herbal material are heat-sensitive, loosing integrity and biological activity by thermal degradation when subjected to heat. The ideal extraction technique is characterized by being versatile, simple, safe, not so expensive, quantitative, non-destructive, and time saving **(Lee et al., 2011; Zhang, Bi and Liu, 2007)**.

1.1. Bioactive compounds and its importance

Bioactive compounds are natural components of plants and plant derived products, being known for their biological properties and effect on human health. There are two major groups of bioactive compounds: essential and non-essential. The essential group comprises majorly vitamins and minerals, responsible for diseases prevention and maintaining specific biochemical processes; while the non-essential bioactive compounds include metabolites such as phenolics, carotenoids, phytosterols, and essential oils, which allow the maintenance of optimal cellular health **(Padayachee et al., 2017)**.

Phenolic compounds are an integral part of human and animal diets, since they represent one of the most numerous and ubiquitous groups of plants metabolites, even though they are considered as anti-nutrients, due to their bad taste **(Padayachee et al., 2017)**. They are identified by their characteristic hydroxyl group (one or more) attached to an aromatic ring, being usually found in plants as esters or glycosides rather than as free compounds **(Vermerris and Nicholson, 2006)**. There are more than 200 different classes already identified, being their classification based (i) on the number of carbons in the molecule, or (ii) on how often they appear in the plant matrixes **(Jun, 2013; Vermerris and Nicholson, 2006)**. These compounds play an important role in plants protection since they are produced as response to external stimulus against pathogenic agents, being able to penetrate the cell membrane of microorganisms, causing its lysis, which will inhibit/delay the oxidation of substrates. Flavonoids are a broad class of secondary plant phenolics with low molecular weight, characterized by the flavan nucleus, with 15 carbon atoms and two aromatic rings linked through a 3-carbon chain **(Vermerris and Nicholson, 2006)**. The different substitution patterns in one of the rings allows flavonoids to have several subclasses, such as flavonols, flavones, flavan-3-ols, flavanones, and anthocyanidins **(Jun, 2013; Trigo et al., 2018)**. Concerning the phenolic acids, they are composed by a single phenolic ring and an organic carboxylic acid, being divided into two classes: the hydroxybenzoic acids and the hydroxycinnamic acids (most easily found in food products).

For so, phenolic compounds are being subject of increasing research due to their beneficial effects in health. Polyphenols are known as natural antioxidants that include catechins, flavanols, flavanones, phenolic acids, and glycosides, which can be easily extracted using water, ethanol, methanol, acetone, or other organic solvents. These extracts show interesting antioxidant and radical scavenging activities, being able to delay or inhibit the oxidation of DNA, proteins and lipids **(Jun, 2013)**. These compounds also play an important role in immune system responses, helping to prevent stress-induced diseases such as melanoma, cardiac disorders, diabetes mellitus, and neurodegenerative diseases as also some cancers **(Alexandre et al., 2018)**. Flavonoids present a cardioprotective effect due to their ability to inhibit lipid peroxidation, chelate redox-active metals, and attenuate other processes involving reactive oxygen species. Moreover, flavonoids exhibit other important physiological activities such as antihypertensive, anti-arthritic, anti-inflammatory, antihepatotoxic, and anti-ulcer actions **(Alexandre et al., 2017d; Vermerris and Nicholson, 2006)**. Phenolic acids are the compounds to which antimicrobial effects are attributed, since their diffusion through cellular membrane leads to the acidification of the cytoplasm and consequent cell death **(Sanchez-Maldonado, 2014)**.

Plant extracts have been recognized for centuries by folk medicine, and the World Health Organization refers that traditional medicine represents the primary health care system for 80% of Asia, Africa, and Latin America populations **(WHO-AFRO, 2010)**. However, the plant species with possible biological activity remain largely unexplored and the effective extraction of natural products continues to play an important role in the drug discovery and development process **(Newman and Cragg, 2016)**.

1.2. Medicinal herbs under study

1.2.1. *Stinging nettle* (*Urtica dioica*)

Urtica dioica L. (stinging nettle) is a common weed, member of the Urticaceae family native to Eurasia, and it is considered a therapeutic herb (**Figure 1.1**). Stinging nettle is usually found in the wildwood, ruins, grassy places, between cultivated plants, and water runnels **(Lahigi et al., 2001; Otles and Yalcin, 2012)**.

Nettle can be used as a drug, food, dye or cosmetic. It has been used for centuries as a traditional medicine for rheumatism, eczema, arthritis, gout, and anaemia, and modern

research has the responsibility to justify its use in traditional medicine, being already proven nettle diuretic, natriuretic, and hypotensive effects **(Orcic et al., 2014)**. There are also other properties acknowledge to nettle leaves such as antioxidant, anti-inflammatory, immune-suppressive and antirheumatoid **(Roschek et al., 2009; Toldy et al., 2009)**. In **Table A1, Appendix A**, there is an extensive review on literature concerning stinging nettle; in **Table 1.1** is possible to consult the most interesting studies.

Figure 1.1. Samples of Stinging nettle

1.2.1.1. Chemical composition of stinging nettle

Nettle biological properties are closely related to its phenolic compound composition such as agglutinin, alkaloids, lecithin, chlorophylls, saccharides (glycogen), phytosterols (cholecalciferol, carnosol), terpenes, fatty acids, among others **(Guil-Guerrero, Rebolloso-Fuentes and Isasa, 2003; Otles and Yalcin, 2012; Roschek et al., 2009)**. Stinging nettle is considered a highly functional and nutritive food **(Adhikari, Bajracharya and Shrestha, 2016)**. Nevertheless, when processed (blanched or cooked) can lose some of its properties. For example, after cooking (98-100 ºC, for 7 min), the content of vitamin A can be significantly decreased, as also the content of protein and saturated fatty acids (can be converted into the monounsaturated and polyunsaturated forms) **(Rutto et al., 2013) (Figure 1.2)**. For so, some authors recommend nettle consumption as a fresh herb, being, this way, supplied 90-100% of vitamin A, dietary calcium, iron, and protein **(Adhikari, Bajracharya and Shrestha, 2016; Rutto et al., 2013)**.

In general, stinging nettle, when fresh, has about 80% of moisture content, and in dry matter, it is constituted by 2.5% fatty substances, 14-17% albumins, 18% protein, 1.2-16% fiber, essential amino acids, vitamins (ascorbic acid and pro-vitamin A), carbohydrates, and minerals (particularly iron and soluble silica) **(Akalın, Karagöz and Akyüz, 2013; Guil-Guerrero, Rebolloso-Fuentes and Isasa, 2003; Hojnik, Škerget and Knez, 2007a;**

Otles and Yalcin, 2012). Relatively to nettle fatty acid content, α-linolenic acid is the most predominant in leaves (40.7%), while seeds are richer in linoleic acid (34.3%) **(Guil-Guerrero, Rebolloso-Fuentes and Isasa, 2003)**, indicating that this plant can be seen as a natural source of n-3 fatty acids.

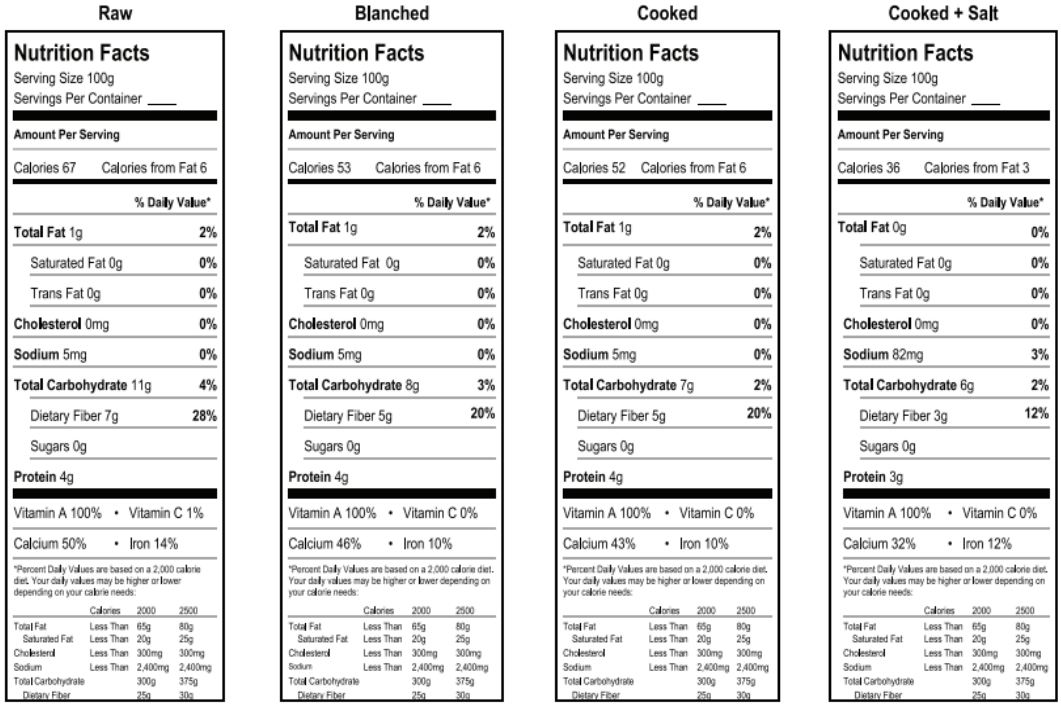


Figure 1.2. Example of food labelling for raw and processed stinging nettle. Adapted from **Rutto et al. (2013)**

The phenolic compounds present in stinging nettle belong to three phenolic classes: hydroxycinnamic acid derivatives (2-*O*-caffeoyl malic acid, chlorogenic acid, *p*-coumaroyl malic acid, caffeic acid), flavonoids (rutin, isoquercetin, kaempferol 3-*O*-rutinoside, isorhamnetin 3-*O*-rutinoside), and anthocyanins (peonidin 3-*O*-rutinoside, rosinidin 3-*O*-rutinoside), being the most abundant the 2-*O*-caffeoyl malic acid (84.1-96.0 mg_{GAE}/g_{dw}), chlorogenic acid (93.9-110.3 mg_{GAE}/g_{dw}), and rutin (44.6-66.0 mg_{GAE}/g_{dw}) **(Pinelli et al., 2008; Vajić et al., 2015)**. The flavonoids are the main class in nettle stalks (31.2-54.4% of total phenolics), being the second class the anthocyanins, present only there (24.4-28.6% of total phenolics) **(Pinelli et al., 2008)**.

Nettle's glandular hairs are composed by formic acid, histamine, acetylcholine, acetic acid, butyric acid, and other irritants, which are responsible for an immediate stinging/burning sensation and skin irritation after direct contact **(Cummings and Olsen, 2011; Roschek et al., 2009)**.

Table 1.1. Studies in literature concerning stinging nettle, its composition, and its biological activities. Adapted from **Table A1** (supplementary material, Appendix A)

	Extraction conditions						Analyses					
Compound	**Extraction method**	**Time (h)**	**Temperature (ºC)**	**Ratio mass to volume (g/mL)**	**Solvent**	**Other extraction methods**	**Antioxidant**	**Anti-microbial**	**Anti-tumoral**	**Other analyses (methods)**	**Results**	**Reference**
Phenolic compounds	-	-	-	1/40	20% Methanol	-	-	-	-	Identification (GC-MS)	-	Kraus and Spiteller (1990)
Chlorophylls a and b; β-carotene; lutein	Supercritical fluid using carbon dioxide SC-CO_2 (20-28 MPa)	2-12	25-60	0.5-2/-	CO_2 and Ethanol	Chloroform; acetone + Sonication	-	-	-	Identification (HPLC)	Extraction yield (24-73 mg/100 g dw)	Sovová et al. (2004)
Bioactive compounds	Stirring	0.25	~100	1/20	Boiling water	-	FRAP; DPPH; ABTS; Superoxide anion radical; Hydrogen peroxide; Metal chelating	Disc diffusion	-	Phenolic compounds (Folin-Ciocalteu); Antiulcer (ethanol-induced ulcer model)	Total antioxidant activity higher for nettle extracts than for control (α-tocopherol); Antimicrobial activity against all tested microorganisms	Gülçin et al. (2004)
Chlorophylls a and b	SC-CO_2 (10-30 MPa)	1	25-60	1/10	Petroleum ether; Hexane	Single step extraction	-	-	-	Identification (HPLC)	Extraction yield (14.3-17.3 g/100 g)	Hojnik, Škerget and Knez (2007a)
Phenolic compounds; Flavonoids	Maceration	72	Room temperature (RT)	1/6.85	70% Ethanol	-	Lipid peroxidation; DPPH	-	-	Phenolic compounds (Folin-Ciocalteu and HPLC)	Total phenolics (7.62 mg GAE/g dw) Flavonoids (1.92 mg quercetin/g dw)	Hudec et al. (2007)
Phenolic compounds	Solid-liquid extraction	-	RT	-	70% Ethanol (pH 3.2)	-	DPPH	-	-	HPLC-DAD; HPLC-MS; HPLC-MS/MS; Fiber content	Main class in stalks are flavonoids and then anthocyanins (only here)	Pinelli et al. (2008)
Flavonol glycosides; Phenolic acids	Solid-liquid extraction	2	RT	3/20	80% Methanol	-	-	-	-	Flavonol glycosides and phenolic acids – Identification (LC-MS); Quantification (RP-HPLC)	High nitrogen levels reduced significantly the concentration of total flavonoids	Grevsen, Frette and Christensen (2008)

Table 1.1. (continued) Studies in literature concerning stinging nettle, its composition, and its biological activities. Adapted from **Table A1** (supplementary material, Appendix A)

Phenolic compounds	Solid-liquid extraction	1	50	1/10	80% Methanol	-	DPPH	-	-	Moisture analysis; Phenolic compounds (Folin-Ciocalteu); Identification (HPLC)	Moisture (81.9/83.1/77.8%); Phenolic compounds (7.62 – 9.91 mg GAE/g dw)	Otles and Yalcin (2012)
Phenolic compounds	Microwave (MW)	0.16	-	1/30	Water	Heating with hot plate	DPPH	-	-	Phenolic compounds (Folin-Ciocalteu and HPLC)	24.64 mg GAE/g	Ince, Sahin and Sumnu (2014)
	Ultrasounds (US)	0.5	-	1/30	Water	Maceration		-	-		23.86 mg GAE/g	
Phenolic compounds	Solid-liquid extraction	48	RT	1/8 or 1/15	80% Methanol	-	-	-	-	Identification (HPLC-MS/MS)	5-*O*-caffeoylquinic acid, rutin and isoquercitrin the most abundant	Orcic et al. (2014)
Phenolic compounds	Maceration	0.63	25	1.25/25	54% methanol	US	DPPH	-	-	Phenolic compounds (Folin-Ciocalteu); Identification and quantification (HPLC; LC-MS)	9.9 mg GAE/g dw	Vajić et al. (2015)
Phenolic compounds	Infusion; Maceration; Decoction	0.5; 24; 0.4	80; RT; 100	1/100	Distilled water	-	FRAP; ABTS	-	Human colon-cancer cells (SW480)	Carbohydrates; Minerals; Phenolic compounds (Folin-Ciocalteu + HPLC)	Higher yield after infusion; cytotoxic and antioxidative properties against cancer cells	Belscak-Cvitanovic et al. (2015)
Phenolic compounds	Infusion	-	-	1/100	Boiling water	Solid-liquid extraction (Ethanol)	FRAP	-	-	Phenolic compounds (Folin-Ciocalteu)	Aqueous extracts with higher concentration of phenolics than ethanolic extracts	Koczka, Petersz and Stefanovits-Bányai (2015)
Bioactive compounds	Soxhlet	6	77	1/20	50% Methanol	Maceration and Soxhlet	DPPH; FRAP; Hydrogen peroxide	-	-	Phenolic compounds (Folin-Ciocalteu); Flavonoids (colorimetry)	Higher phenolics concentration after UAE and higher flavonoids concentration after Heat reflux	Stanojević et al. (2016)
	Heat reflux	2	25	1/20	30, 50, 80, 100% Methanol							
	US assisted extraction (UAE)	1	25	1/20	50% Methanol							
Bioactive compounds	Solvent	24	-	-	Dichloro-methane	-	-	-	HCT-116 cells	DNA fragmentation (TUNEL assay); RNA extraction; rt-PCR; Cytotoxicity (MTT)	Dichloromethane extracts have potential for antiproliferative activity against HCT-116 colon cancer cells	Mohammadi et al. (2016)

Table 1.1. (continued) Studies in literature concerning stinging nettle, its composition, and its biological activities. Adapted from **Table A1** (supplementary material, Appendix A)

Phenolic compounds	Solid-liquid extraction	0.5-2	25-65	1/40	50-100% Ethanol	-	FRAP; CUPRAC	-		Phenolic compounds (Folin-Ciocalteu); Anti-aging (collagenase and elastase)	Antiaging effect by inhibition of enzyme activities (elastase and collagenase) due to the presence of ursolic acid and quercetin	Bourgeois et al. (2016)
Chemical profile	Hydro-distillation	2	-	40g	Water (and petrol ether)	-	-	-	-	Chemical analysis (GC-MS-FID)	12 monoterpenes detected (3 quantified)	Đurović et al. (2017)
	Soxhlet	-	-	1/30	96% Ethanol	-	-	-	-	Chlorophylls and carotenoids	Extraction yield (21.75%)	
	UAE	1	45	1/30	96% Ethanol	-	-	-	-	Fatty acids (GC-FID); elemental analysis (ICP-OES and mercury analyser)	Extraction yield (3.65%)	
Bioactive compounds	UAE	0.75	-	1/30	Water	-	DPPH; reducing power; direct current polarographic assay	MIC; antifungal activity	-	Phenolic compounds (Folin-Ciocalteu and UHPLC-DAD-HESI-MS/MS); Flavonoids (colorimetry); Cytotoxicity (MTT assay)	Phenolics (463.6 mg CAE/g); Flavonoids (11.00 mg CE/g); DPPH (16.93 ug/mL)	Zekovic et al. (2017a)
	MAE	0.75	-	1/30	Water	-						
	Subcritical H_2O	0.75	-	1/30	Water	-						
Bioactive compounds	Solid-liquid extraction (in water bath)	1	70	-	70% Ethanol	-	-	Disc diffusion; MIC	-	Phenolic acids, flavonoids, flavones and flavonols (HPLC-DAD/Vis); Phenolic compounds (Folin-Ciocalteu); Flavonoids (colorimetry)	Major compound (Caffeic acid, 163.01 ± 3.63 µg/g); Phenolics (25.85 ± 1.2 mg GAE/g); Flavonoids (22.47 ± 0.7 mg CAE/g)	Zenão et al. (2017)
Bioactive compounds	Boiling; sonication	0.5; 0.25	~100	1/20	Hot water and ethanol	-	FRAP; DPPH	-	-	α-amylase, α-glucosidase and formation of advanced glycation end products (AGE) inhibition assays in vitro; Phenolic compounds (Folin-Ciocalteu and UPLC-MS/MS)	Total phenolic compounds (27.7 mg GAE/L)	Sekhon-Loodu and Rupasinghe (2019)

1.2.1.2. Biological activities of stinging nettle

Stinging nettle has a recognized antioxidant activity, majorly related to its total phenolic content. Various phenolic compounds were identified in methanolic extracts of nettle (roots, stalk, and leaves), and it was reported a higher antioxidant activity when leaves were used (76.06 mg_{GAE}/g_{dw}), than stalks (37.56 mg_{GAE}/g_{dw}), or roots (9.86 mg_{GAE}/g_{dw}) **(Lahigi et al., 2001; Otles and Yalcin, 2012)**. These authors have also reported that when in comparison with dried nettle, fresh samples had higher phenolic content, indicating that its consumption while fresh could be healthier. Similar results were also reported by **Orcic et al. (2014)**, who found that methanolic extract is a rich source of 5-*O*-caffeoylquinic acid (also known as chlorogenic acid), rutin and isoquercitrin, confirming the potential of nettle for antioxidant, DNA-protective, anti-inflammatory, antihypertensive, anti-microbial, and analgesic properties **(Akalın, Karagöz and Akyüz, 2013)**.

Nettle also revealed great potential for anti-inflammatory activity. For example, **Toldy et al. (2009)** studied the combined effect of exercise (swimming) and nettle leaves supplementation in rats with brain damage. The results showed that nettle consumption decreases the reactive oxygen species content, and improves the activation of nuclear factor kappa B (NF-κB, an inflammatory transcription factor), allowing to decrease the inflammatory process and prevent neurodegenerative diseases **(Toldy et al., 2009)**. Similar results had been already reported by **Riehemann, Behnke and Schulze-Osthoff (1999)** after nettle leaves extract supplementation to human and mouse cell lines, suggesting that these extracts interfered with a common target in the NF-κB pathway. NF-κB activation or inhibition can be related to the action of flavonoids or phenolic acids, such as caffeic acid, quercetin and curcumin, by antioxidant mechanisms **(Riehemann, Behnke and Schulze-Osthoff, 1999)**. It is noteworthy that extracts obtained with water or methanol were not able to reduce inflammation, being necessary to elucidate which are the bioactive compounds responsible for these activity, in order to optimize the dosage and route of delivery **(Johnson et al., 2013)**.

Concerning antimicrobial activity, stinging nettle is also efficient on bacterial inactivation, but has some problems against fungal species, possibly due to its thicker cell wall with a higher percentage of chitin **(Modarresi-Chahardehi et al., 2012)**. Nevertheless, pathogenic microorganisms presented low values of minimum inhibitory

concentration (MIC), and the extracts obtained with a polarity gradient (hexane, chloroform, ethyl acetate, and methanol) showed better antimicrobial activity, mostly against Gram-positive bacteria **(Modarresi-Chahardehi et al., 2012)**. Also when nettle was added to meat in order to produce sausages, a significant decrease on microbial loads of total aerobic mesophilic bacteria, lactic acid bacteria, Enterobacteriaceae, *Micrococcus*/*Staphylococcus*, and yeast and moulds was observed, indicating a great potential of nettle extracts for microbial growth inactivation activity **(Aksu and Kaya, 2004)**.

Another positive use of nettle extract is its potential efficacy as antitumoral. When compared to butylated hydroxyanisole (BHA), a daily dosage of ethanolic nettle extract (50-100 mg/kg body weight for 14 days) had positive effects on biotransformation of some enzyme systems and antioxidant enzymes **(Ozen and Korkmaz, 2003)**.

1.2.1.3. Extraction methods used on stinging nettle

Nettle compounds are usually extracted using conventional or supercritical fluid extraction (SC-CO_2) (see **Table 1.1**). For example, **Akalın, Karagöz and Akyüz (2013)** studied the optimization of phenolic compounds extraction from nettle using supercritical ethanol as extraction technique (336 ºC, 144 min, with a plant concentration of 14%), being the obtained yield of 45.3% dw. In another study, chlorophylls a and b (the two major pigments in nettle), β-carotene, and lutein were extracted (73, 100, 24, and 39 mg/100 g_{dw}, respectively) from stinging nettle using SC-CO_2 with ethanol **(Hojnik, Škerget and Knez, 2007a; Hojnik, Škerget and Knez, 2007b; Sovová et al., 2004)**, being the maximum yields obtained with SC-CO_2 similar for carotenoids and lower for chlorophylls in comparison with classical extraction techniques using chloroform and acetone **(Sovová et al., 2004)**. Similar results were reported by **Sajfrtová et al. (2005)** who verified that SC-CO_2 allowed an efficient extraction of β-sitosterol and scopoletin from stinging nettle, with yields (0.63 mg/g_{dw} and 0.058 g/g_{dw}, respectively) higher than ultrasonic extraction with diethyl ether (0.26 and 0.016 mg/g_{dw}, respectively).

1.2.2. *Winter Savory* (*Satureja montana* L.)

Satureja montana L. is commonly known as winter savory or mountain savory, and belongs to the Lamiaceae family; it is a perennial herb usually found in the Mediterranean

area, such as the southern Europe and the North of Africa, and likes to grow in arid, sunny, and rocky regions (**Figure 1.3**) **(Coutinho de Oliveira et al., 2012; Grosso et al., 2009b; Slavkovska et al., 2001; Zeljković et al., 2015)**. Savory is a plant often used in traditional medicine being also commonly used as an aromatic herb as a spice for food and tea, mainly for seasoning of meat and fish, as well as a flavouring for soup, sauces or canned food **(Coutinho de Oliveira et al., 2012; Gião et al., 2009)**. In **Table A2, Appendix A**, there is an extensive review on literature concerning winter savory; in **Table 1.2** it is possible to consult the most interesting studies.

Figure 1.3. Samples of winter savory

1.2.2.1. Chemical composition of winter savory

Winter savory can appear in the wild under a wide variability of morphologies even in same population, which can cause misunderstanding from taxonomic perspective. These variations of appearance are consequence of alteration in the content and chemical composition between populations. Nevertheless, at least for the essential oil characterization, there is already an International Standard (ISO 7928-1-1991), stating that the minimum yield of essential oil (on a dry basis) required for springs of *S. montana* is 0.3% and it must contain *γ*-terpinene, *p*-cymene, linalool, 1-terpinen-4-ol, and carvacrol as main constituents **(Hajdari et al., 2016; Sefidkon, Abbasi and Khaniki, 2006)**. The whole plant can be used for their medicinal benefits especially upon the digestive system, since this herb is rich in several biologically active constituents such as essential oils, tannins, triterpenes and flavonoids **(Coutinho de Oliveira et al., 2012; Fraternale et al., 2007; Grosso et al., 2009b; López-Cobo et al., 2015)**. The identified major volatile constituents and their relative percentages are terpinene, ranging from 4.9 to 10.6%, carvacrol 13.7–52.4%, *p*-cymene 3.0–11.8%, and thymol between 9.92–45.2% **(Vladić et al., 2017)**.

Table 1.2. Studies in literature concerning winter savory, its composition and biological activities. Adapted from **Table A2** (supplementary material, Appendix A)

	Extraction conditions						Analyses					
Compound	**Extraction method**	**Time (h)**	**Temperature (ºC)**	**Ratio mass to volume (g/mL)**	**Solvent**	**Other extraction methods**	**Antioxidant**	**Anti-microbial**	**Anti-tumoral**	**Other analyses (methods)**	**Results**	**Reference**
-	Solid-liquid extraction	48	RT	1/100	70% Methanol	-	DPPH	-	Sulforhodamine B assay (cell lines HeLa, MCF-7, HT-29)	-	Antiproliferative effect on HeLa cell line with IC_{50} ranging from 0.41 to 0.84 mg/mL	Cetojevic-Simin et al. (2004)
Bioactive compounds	Maceration	2x24	RT	1/25	70% Methanol	-	Hydroxyl radicals by Fenton reaction	Disc diffusion and microbroth dilution	-	Consecutive extractions with Petroleum ether, Chloroform, Ethyl acetate, and *n*-Butanol	*n*-Butanol extract had the best antioxidant activity (100% at 0.5 mg/mL in Fenton reaction system	Ćetković et al. (2007a)
Phenolic compounds	Maceration	2x24	RT	10/2x20	70% Methanol; Petroleum ether, Chloroform, Ethyl acetate, and *n*-Butanol	-	-	-	-	Phenolic compounds (Folin-Ciocalteu and HPLC); Sequential extractions	Higher concentration of total phenolic in ethyl acetate (47.59 mg/g) and *n*-butanol (96.70 mg/g)	Ćetković et al. (2007b)
Volatile fraction	SC-CO_2 (90 bar)	-	40	100/-	CO_2 (1.1 kg/h)	Hydro-distillation	-	MIC; MLC	-	Identification (GC-MS)	Growth inactivation of *Bacillus subtilis* and *Bacillus cereus*, *Botrytis* spp. and *Pyricularia oryzae*	Silva et al. (2009)
Non-volatile fraction	SC-CO_2 (250 bar)	4	´-	5/-	CO_2	Soxhlet	-	-	-	Alzheimer's disease (Ellman's assay); Identification and quantification (HPLC-DAD)	High content of catechin, chlorogenic, vanillic, and protocatechuic acids; Inhibition of butyrylcholinesterase	
Non-volatile fraction	Maceration	72	20	1.5/10	Ethanol and water	Soxhlet	DPPH; FRAP	Disc diffusion; MIC	-	Phenolic compounds (Folin-Ciocalteu); Identification and quantification (GC-MS)	Major volatile constituents of the essential oil were carvacrol (306 g/L), and thymol (141 g/L); Higher antioxidant activity for hot water extracts	Serrano et al. (2011)
Volatile fraction	Hydro-distillation	3	-	1/7	Deionised water							

Table 1.2. (continued) Studies in literature concerning winter savory, its composition and biological activities. Adapted from **Table A2** (supplementary material, Appendix A)

Bioactive compounds	Ultrasonic bath	0.5	-	1/10	Ethanol	-	DPPH; total antioxidant capacity	-	-	Alzheirmer's disease (Ellman's assay); Identification and quantification (RP-HPLC)	Rosmarinic acid was the predominant constituent; High antioxidant activity; 75% inhibition at 1 mg/mL on Ellman's colorimetric assay	Vladimir-Knežević et al. (2014)
Rosmarinic acid	Maceration	0.083	-	1/100	Boiling water	-	ABTS; ORAC	-	-	Structural changes (SEM; DSC); Identification (HPLC)	Individual and small sizing chitosan nanoparticles were obtained	da Silva et al. (2015)
Phenolic compounds	Solid-liquid extraction	-	-	1/10	Methanol, ethanol, acetone	-	DPPH; ABTS	-	-	Phenolic compounds (Folin-Ciocalteu and HPLC–DAD–ESI–TOF–MS)	42 compounds identified for the first time; Chlorogenic acid was the most abundant compound	López-Cobo et al. (2015)
Phenolic compounds	Soxhlet	-	-	200/-	Chloroform and methanol	-	DPPH; ABTS; FRAP; Molybdenum ions; Metal-chelating	-	-	Phenolic compounds (Folin-Ciocalteu); Flavonoids (colorimetry); Phenolic acids (HPLC–UV)	Source of natural phenolic compounds, with significant antioxidant activities	Zeljković et al. (2015)
Essential oil	SC-CO_2 (100-300 bar)	4	40	50/-	CO_2 (0.2 kg/h)	-	DPPH	-	Human cancer cell lines (HeLa, MDA-MB-453, K562) and normal cell lines MRC-5 (MTT assay)	Moisture; Identification (GC-MS and GC-FID)	Carvacrol as the most concentrated compound	Elgndi et al. (2017)
Bioactive compounds	Subcritical water extraction	0.35	200	1/10	Water	-	DPPH	-	-	Phenolic compounds (Folin-Ciocalteu); Flavonoids (colorimetry); Volatile fraction (GC-MS)	Total phenols, total flavonoids, and IC_{50} obtained were 11.24 g/100 g, 6.84 g/100 g and 0.0028 mg/mL, respectively	Vladić et al. (2017)
Phenolic compounds	Microwave	0.016-0.42	-	1/10	70% Ethanol	-	DPPH; reducing power	-	-	Phenolic compounds (Folin-Ciocalteu); Flavonoids (colorimetry)	Microwaves proved to be suitable for fast and effective extraction of total phenolics	Zekovic et al. (2017a)
Essential oil	Hydro-distillation	-	-	-	-	-	-	MIC; MBC	-	GC-MS	Antimicrobial activity is attributed to the presence of carvacrol	Babaei et al. (2018)

1.2.2.2. Biological activities of winter savory

S. montana has several biological properties related to its rich and diverse composition of secondary metabolites. Its extracts have several biological activities, such as anti-inflammatory, antimicrobial, antifungal, antioxidant, antiproliferative and anti-cholinesterase **(Ćetković et al., 2007a; Elgndi et al., 2017; Silva et al., 2009).**

Silva et al. (2009) studied the possibility of supercritical fluid extraction (SFE) help to obtain extracts able to potentially control Alzheimer's disease, compared to conventional extractions as hydrodistillation and Soxhlet. The activities of the two indicator enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase, were assessed for all the extracts. The results showed not only a higher concentration of the interesting compounds, but also a selective and significant butyrylcholinesterase inhibition after supercritical extraction for the extracts with the non-volatile fraction **(Silva et al., 2009)**. Similar results were reported by **Vladimir-Knežević et al. (2014)** who showed that ethanolic extracts at 1 mg/mL of *S. montana* are able to effectively inhibit AChE activity, mainly due to their content in hydroxycinnamic acids, specially the rosmarinic acid. These findings indicate that species belonging to the Lamiaceae family are a potential rich source of various natural AChE inhibitors that could be useful in the prevention of Alzheimer's and other neuro-related diseases **(Vladimir-Knežević et al., 2014)**

Winter savory can also be used for skin and mucous inflammation, when treated externally, and can also act as stimulant aphrodisiac and as a treatment for premature ejaculation **(Stanojkovic et al., 2013; Zavatti et al., 2011)**. **Tampieri et al. (2005)** and **Fraternale et al. (2007)** reported the antifungal activity of *S. montana*, while **Ciani et al. (2000)** reported that 46 species of yeasts were inhibited with a low concentration of savory essential oil; **Skocibusic and Bezic (2004)** investigated its activity against Gram-positive and Gram-negative bacteria and correlated its high effect to the presence of phenolic compounds. Moreover, **Yamasaki et al. (1998)** reported that *S. montana* aqueous extract showed a potent anti-HIV-1 activity. **Skocibusic and Bezic (2004)** also studied the effect of savory essential oil (100 g of dried plant material subjected to a 3 h hydrodistillation) on the inactivation of Gram-positive bacteria, Gram-negative bacteria, two yeasts and one filamentous fungus. After obtaining MIC (minimum inhibitory concentration) and MBC (minimum bactericidal or fungicidal concentration) values, the main results showed that savory essential oil presented high antimicrobial activity against *Candida albicans* and

Aspergillus fumigatus, as also against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium* and multidrug resistant *Serratia marcescens*. The authors attributed this effect to the correlation between high antimicrobial activity and the presence of phenolic components, such as carvacrol, eugenol, and thymol **(Skocibusic and Bezic, 2004)**.

Several studies report the antioxidant activity of the essential oil and volatile compounds of *S. montana* **(Grosso et al., 2009a; Radonic and Milos, 2003; Zeljković et al., 2015)**; nevertheless, only few studies focus on its phytochemical composition, as well as on the antioxidant and antimicrobial activities of the non-volatile compounds **(Serrano et al., 2011)**, allowing the development of new and interesting research on this herb. When comparing the essential oil with ethanolic extracts, it was observed that while the essential oil had the highest antimicrobial activity against several bacteria tested, the ethanolic extract did not reveal activity only against *Salmonella typhimurium* **(Serrano et al., 2011)**.

1.2.2.3. Extraction methods used on winter savory

Hydrodistillation (HD) and steam distillation are the most common methods used to isolate plant volatile components; nevertheless, thermal degradation and hydrolysis of other compounds are some of the disadvantages. These limitations are usually overcome by using supercritical fluid extraction since it can operate at moderate temperatures and there are no hydrolysis reactions **(Grosso et al., 2009b)**. Supercritical fluid extraction (SFE) of the volatile oil from savory was performed under different conditions of pressure (90 and 100 bar), temperature (40 and 50 ºC), particle size (0.4, 0.6 and 0.8 mm), and CO_2 flow rate (0.8, 1.1 and 1.3 kg/h) in order to obtain optimal conditions for this extraction. The results were compared with those obtained for the essential oil isolated by HD. The main obtained compounds were carvacrol (52.2–62.0% for HD *vs*. 41.7–64.5% for SFE), thymol (8.6–11.0% for HD *vs*. 6.0–11.3% for SFE), *p*-cymene (6.9–12.8% for HD *vs*. 6.0–17.8% for SFE), *c*-terpinene (6.4–9.4% for HD *vs.* 2.3–6.0% for SFE) and *b*-bisabolene (2.0–2.7% for HD *vs.* 2.2–3.5% for SFE). As can be observed in the results, the major difference between SFE and HD extractions was the relative amount of thymoquinone, an oxygenated monoterpene with important biological activities **(Grosso et al., 2009b)**. Similarly, after SFE at temperatures ranging from 25 to 80 ºC and pressures up to 30.0 MPa, **Coelho et al. (2012)** reported that the major difference between HD and SFE oils

was, again, the relative amount of thymoquinone. Also **Silva et al. (2009)** have compared conventional (HD and Soxhlet) with alternative (SFE) extraction method in order to assess the best technique to obtain bioactive compounds from savory. The comparison was performed between two different extracts: the volatile fraction (SFE at 90 bar *vs.* HD) and the non-volatile fraction (SFE at 250 bar *vs.* Soxhlet extraction). The main results showed that the extracts obtained after SFE showed not only a higher content of several different compounds, such as β-catechin, chlorogenic, vanillic, and protocatechuic acids, as also significantly inhibited butyrylcholinesterase, whereas the conventional extract did not affect this enzyme **(Silva et al., 2009)**. Also **Rezvanpanah et al. (2008)** compared the effectiveness of conventional HD with microwave-assisted hydrodistillation in order to obtain essential oils from savory. Although the extraction yields obtained for both extraction methods were similar (~0.7%, w/w), the results showed that microwave extraction occurred in a shorter time than the conventional method (90 and 180 min, respectively), being a potential alternative method **(Rezvanpanah et al., 2008)**. Nevertheless, these new extraction methods must be optimized, and experimental designs, such as Box-Behnken, are usually performed. **Zekovic et al. (2017b)** optimized the extraction by microwave assisted extraction of savory bioactive compounds by maximization of total phenolics, total flavonoids, and antioxidant activity of ethanolic extracts, underneath the variation of three independent variables (extraction time, ethanol concentration, and irradiation power). The main results showed that the optimal conditions predicted were an extraction time of 27.5 min, ethanol concentration of 55.8%, and an irradiation power of 632 W, allowing to obtain optimal values of total phenolics and total flavonoids of 6.87 g_{GAE}/100 g_{dw} and 4.48 g_{CAE}/100 g_{dw}, respectively, while the IC_{50}, and EC_{50} values for DPPH assay and reducing power assay would be 3.20 μg/mL and 9.83 μg/mL, respectively, with a general desirability of 0.918 **(Zekovic et al., 2017b)**.

1.2.3. Case study – addition of an herbal extract to a food product

World Health Organization recommends a minimum daily intake of fruit and vegetables of 400 g in order to prevent chronic diseases and micronutrient deficiencies **(WHO, 2017; WHO, 2013)**. Beverages are a practical and easy way of increasing the consumption of bioactive compounds, and carrot juice is a vegetable juice consumed by many **(Reuters, 2019)**. Nevertheless, when compared to other vegetable juices available in

the market (such as tomato or beetroot), carrot juice has the lowest concentration of phenolic compounds and antioxidant activity **(Wootton-Beard, Moran and Ryan, 2011)**. For so, there is the possibility to modify the traditional carrot juice with an extract rich in bioactive compounds, such as phenolic acids and flavonoids. Another limitation of raw carrot juice is the fact that its natural characteristics, such as a pH close to neutral (ranging from 6.0 to 6.5) and a high water activity value (aw>0.85), present no natural hurdles against the growth of spoilage nor pathogenic microorganisms, making its shelf-life of only 1-2 days when stored under refrigeration **(Pilavtepe-Çelik, 2013)**. To overcome this limitation, the food industry adds acids to lower its pH or uses thermal processing to inactivate the microbial growth **(Reyes-De-Corcuera et al., 2014)**. Nevertheless, thermal treatments could destroy nutrients, such as vitamin C and β-carotene, and adversely affect organoleptic properties, being needed the application of other processing techniques such as high hydrostatic pressure pasteurization, since it does not use high temperatures, making it possible to effectively inactivate microorganisms and enzymes and preserve nutritional/functional and sensorial characteristics of the products **(Considine et al., 2008)**.

1.3. Extraction

Extraction is the first step for the recovery (isolation and purification) of important bioactive components present in herbal materials, and it can be described as a mass transport phenomenon where solids present in a plant matrix are transferred into the solvent up to their equilibrium concentration **(Lee et al., 2011)**. The major problem is that those bioactive compounds are enclosed in insoluble structures (vacuoles of plant cells or lipoproteins bilayers), making its extraction very complicate **(Corrales et al., 2008)**. Several compounds in plant/herbal material are heat-sensitive, losing integrity and biological activity by denaturation when subjected to heat.

The ideal extraction technique is characterized by being versatile, relative simple, safe, not so expensive, quantitative, non-destructive, and time saving **(Lee et al., 2011; Zhang, Bi and Liu, 2007)**. The aim of an extraction process is to provide the maximum yield and the highest quality in terms of concentration of the target compounds, and usually consists in two consecutive steps: (i) mixture of material with solvent, for swelling and hydration, and (ii) movement of soluble compounds into the solvent and its consequent diffusion and extraction **(Huang et al., 2013; Shah, Bosco and Mir, 2014)**. The extraction technique

strongly influences the composition of the extract, and there are many techniques to recover interesting compounds from plants, such as the conventional techniques (Soxhlet extraction, maceration, heat reflux, agitation, boiling, and distilling) **(Huang et al., 2013)**.

1.3.1. Conventional extraction methods

In industry, the most used extraction method is the solid-liquid extraction, which consists on an unstable-state of mass transfer of several compounds from a solid matrix to an appropriate solvent. When the solvent gets in contact with the tissues, the compounds should pass through the space between the cells, or through the holes formed in the cell wall, being transported from the matrix to the extraction solvent by internal/external diffusion processes (from a region of high concentration to that of lower concentration according to the concentration gradient) (**Figure 1.4**) **(Alexandre et al., 2017a; Huang et al., 2013)**.

Soxhlet extraction is known as the principal reference for evaluating the performance of solid–liquid extraction methods. This methodology bests other conventional techniques in performance, except for the extraction of thermo-sensitive compounds since it needs high temperatures to be efficient and it needs long extraction times, since the process is repeated as many times as necessary until the extraction is completed **(Wang and Weller, 2006)**. Maceration is also one of the most used conventional techniques and consists in the breakage of the cell walls by crushing the material in order to reduce the particle size, and then soak it in a solvent; the minor the particle size, the greater the contact area between the particles and the solvent. Although this process can occur at room temperature, avoiding thermo-sensitive compounds degradation, since it only depends on agitation in order to improve the extraction process, it can take several hours or days to be complete **(Azmir et al., 2013)**.

The conventional solvent extraction methodology represents 80% of the total processing time, 90% of the required energy, and more than 99% of the solvent used for the whole analysis procedure **(Alexandre et al., 2017a)**. Thus, one may add an analytical procedure for the extraction process, the evaporation of the solvent by distillation for concentration or purification, and the analysis of the compounds (which may be done in a few minutes or seconds), the whole process can take at least several hours or even days). These conventional techniques are mostly based on the choice of one correct solvent, the

use of mild/high temperatures (60-90 ºC) that causes thermal degradation, and agitation in order to increase the solubility of materials and mass transfer rate, being reflected on long extraction times, high costs, and low extraction efficiency **(He et al., 2011)**.

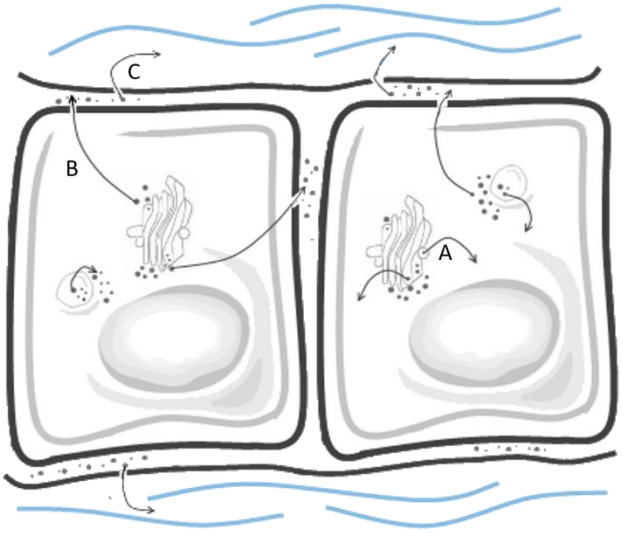


Figure 1.4. Scheme of a solid–liquid extraction. A represents the internal diffusion process; B represents the external diffusion process; and C represents the solvent extraction process. Adapted from **Alexandre et al. (2017a)**

Due to these limitations and several more, such as high costs, high organic solvent consumption, and environmental pollution of the conventional methods, it has been necessary to develop new extraction methods, such as microwave, ultrasounds, supercritical fluids (using mostly CO_2), and high hydrostatic pressure assisted extractions (HPE) **(Huang et al., 2013)**. Nevertheless, some of those new technologies still need temperature control (such as microwave assisted extraction), are restricted to the solvents to use, and are very time consuming **(Shouquin et al., 2006)**. On the other hand, HPE can operate at refrigerated or room temperatures, ensuring that compound denaturation is avoided, easing the extraction of such components. For so, HPE methodology enable the extraction of heat-sensitive compounds, without major damage and denaturation, and has been recognised as an environment-friendly technology by the Food and Drug Administration **(US-FDA, 2000)**.

1.3.2. High hydrostatic pressure

The effects of high hydrostatic pressure in biotechnology have been largely studied in the last decades, being already successfully applied in the processes of pasteurization for

gentle food preservation and pharmaceutical compounds processing. High hydrostatic pressure processing (HPP) follows two principles: the isostatic rule, that states that pressure is instantaneously and uniformly transmitted through food, regardless of its size, shape, if it is in direct contact with compression media or in a flexible package **(Smelt, 1998)**; and the Le Chatelier's principle, that states that when an equilibrium system is perturbed, it tends to respond in order to minimize the perturbation. So, any phenomenon accompanied with a volume decrease is favoured by pressure, and *vice-versa* **(Smelt, 1998)**.

The major advantages of HPP rely on the fact that it enables food processing at low or room temperatures, it causes microbial inactivation, leading to extended shelf-life of different food products, and it can be used for new ingredients development, with new functional and bioactive properties **(Rastogi et al., 2007)**. Nonetheless, the cost of equipment's is still the major obstacle faced by HPP application in industry, being necessary to develop equipment with a new design, for various products, and with different applications besides food processing.

As stated above, one of the major advantages of HPP is the fact that it does not need to use high temperatures, making it possible to effectively inactivate microorganisms and enzymes and still preserve nutritional/functional and sensorial characteristics of food products **(Considine et al., 2008)**. Nevertheless, it can cause some structural changes in structurally-fragile materials, such as cell deformation, cell membrane damage, and protein partial denaturation, since during the compression phase, through adiabatic heating, the temperature can rise about 3 ºC per 100 MPa, depending on the biomaterial matrix (for example, if the food is rich in fats, such as butter or cream, the temperature increase is greater, being about 8 ºC per 100 MPa) **(Knorr et al., 2011; US-FDA, 2000)**. Nevertheless, during the decompression phase, the pressurized material can cool down to their original temperature if no heat is lost to, or gain from, the walls of the pressure vessel **(Rastogi et al., 2007; US-FDA, 2000)**. Besides microorganism's inactivation, the pressure level used for food processing (100-800 MPa) can act in food constituents by protein denaturation/modification, enzymes activation/inactivation and alterations on carbohydrates and lipids. As HPP only acts in noncovalent bonds (ionic, hydrophobic and hydrogen bonds) and does not majorly affect covalent bonds, it only causes alterations in larger molecules, such as proteins and lipids by alteration of their secondary, tertiary and

quaternary structures, leaving low molecular weight compounds intact, such as peptides, vitamins, and flavour and pigmentation compounds **(Linton and Patterson, 2000; Rastogi et al., 2007)**.

1.3.3. High pressure extraction

High pressure extraction (HPE) follows the same principles of HPP, abide by isostatic principle and uses low or room temperatures, at pressure that ranges from 100 to 800 MPa. It follows two principles: according to mass transfer theory, the rate of mass transfer equals to pressure by resistance of mass transfer (*i.e.* pressurized cells show increased permeability) **(Yan, 2002)**; and based on phase behaviour theory, the dissolution is faster at higher pressure levels **(Sadus, 2012)**. For so, HPE methodology enables the extraction of heat-sensitive compounds, without major damage/denaturation, and has been recognised as an environmentally-friendly technology by the Food and Drug Administration **(US-FDA, 2000; Xi, 2006b)**. Another great HPE advantage is the possibility of combining different solvents (and solvent ratios), with distinct polarities, allowing the extraction of different compounds (strong, weak, polar, non-polar, etc.), as well as to manipulate the amount of impurities present in the final extract **(Shouqin, Junjie and Changzhen, 2004)**. The two most used solvents are water and ethanol (alone or in mixture) due to their different polarities and the facility of evaporate and recycle the ethanol (which, when compared to other organic solvents is considered non-toxic and not expensive) **(Shouqin, Xi and Changzheng, 2005)**. For example, ethanol was chosen in detriment of methanol, chloroform, and *n*-butanol due to its characteristics, even though the others presented higher extraction yields **(Chen et al., 2009; Prasad et al., 2009d; Prasad et al., 2009e; Shouqin, Xi and Changzheng, 2005; Xi, 2006a)**.

HPE presents other advantages comparatively to conventional extraction techniques, such as (i) short time processing (the differential pressure between the inner and outer cell is very large, allowing the solvent to permeate through the broken cells very quickly), (ii) low energy consumption, (iii) high solubility when under high pressure, (iv) high amount of solvent inside the cell, leading to easier permeation due to wall and membrane breakage, causing a high rate of mass transfer and consequent high extraction yield **(Shouqin, Junjie and Changzhen, 2004)**. The damages caused in cell membranes are consequence of the appearance of hollow openings, development of smaller particles from the broken plant

tissue, etc. as can be seen by scanning electron microscopy of ginseng samples **(Chen et al., 2009; Qadir et al., 2009)** and light and scanning electron microscopy of dried pollen grains **(Altuner, Çeter and Alpas, 2012)** after HPE.

HPE comprehends three principal steps (**Figure 1.5**): (1) Pressure boost stage: includes the mixture of the raw material with the solvent, the time until achieving the target pressure inside the vessel, and the consequent equilibrium between inside and outside the cell (very short); (2) Pressure maintaining stage: treatment under high pressure (100-800 MPa) for a determined period of time; and (3) Pressure relief stage: quick pressure release (from target pressure to atmospheric pressure in only a few seconds) that causes cell expansion and fluid circulation, resulting in significant cell and membrane damage (leading to higher permeation), followed by concentration/purification of the compounds of interest **(Huang et al., 2013; Shouqin, Xi and Changzheng, 2005)**.

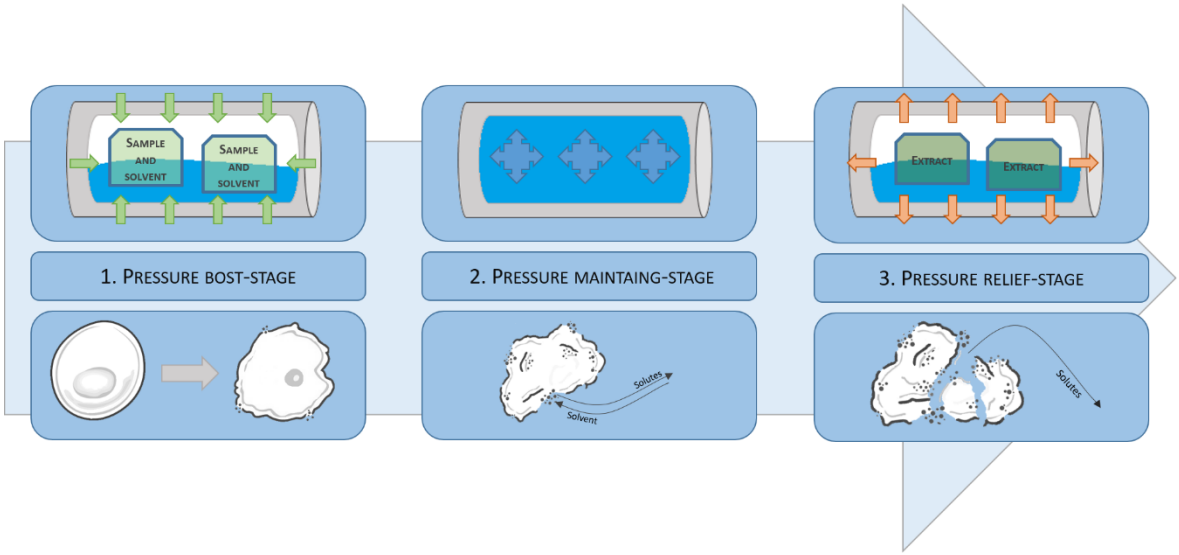


Figure 1.5. Scheme of an extraction process assisted by high pressure. Adapted from **Alexandre et al. (2017a)**

The different parameters to take in consideration for HPE are, in order of importance: extraction temperature, pressure level, solvent and its concentration, ratio of solvent to raw material, and pressure holding time **(Chen et al., 2009)**. The extraction temperature is of extreme importance due to the presence and efficient extraction of thermo-sensitive compounds. A rise in temperature can break the phenolic-matrix bonds and influences the membrane structure of plant cells making them less selective by coagulation of lipoproteins **(Prasad et al., 2009e).**

The pressure level can vary according to the compounds to extract, and was observed that the higher the hydrostatic pressure, the more solvent can enter cells and consequently, the more compounds can permeate out to the solvent **(Xi et al., 2009)**. Also, the solvent choice (and its concentration) is closely related to the components to extract; it should be non-toxic and easy to evaporate from the final extract. The ratio of solvent to raw material is another important parameter to take into account, since the dissolution of bioactive components into the solvent is a physical process, and when the solvent volume is high enough, there is a much higher probability to enter in contact with the interest compounds, leading to higher extraction yields. Finally, the pressure holding time is the time necessary to form the equilibrium of solvent concentration between the inside and outside of the cells and to the solvent to get into full touch with the components to extract **(Xi et al., 2009)**.

Recently, many studies have come to light about the use of HPE (see **Table A3, Appendix A**), especially on fruits and vegetables (such as extraction of carotenoids from orange juice and strawberry puree), herbs **(Table 1.3)** (for example, ginsenosides from ginseng and polyphenols from green tea), food by-products (such as phenolic compounds from longan fruit pericarp and anthocyanins from grape skins), and other foods (as extraction of flavonoids from propolis) **(Huang et al., 2013)**.

1.3.3.1. High pressure assisted extraction of bioactive compounds

The first study regarding HPE was developed by **Shouqin, Junjie and Changzhen (2004)** who reported some pilot works in order to demonstrate its applicability. The authors verified that different HPE conditions led to extracts with different colour, being the one with fewer impurities (more transparent and limpid) the one extracted at 600 MPa for 5 min, using water as solvent. Another example is the extraction of chlorophylls, since an ethanol concentration lower than 50%, a pressure level lower than 400 MPa, and an extraction time lower than 10 min led to no chlorophyll extraction, since these conditions do not destroy the chloroplast membranes **(Shouqin, Junjie and Changzhen, 2004)**. The same research group reported the best conditions for ginsenosides extraction from *Panax ginseng* through mono-factor experiments (for solvent (water, ethanol, methanol, or *n*-butanol), solvent concentration (10-90%), ratio mass to solvent volume (1:10, 1:25, 1:50, 1:75 or 1:100), extraction pressure (100-600 MPa), and extraction time (1-5 min)) **(Shouqin, Ruizhan and Changzheng, 2007)**.

Table 1.3. High pressure assisted extraction studies in literature; focus on herbs and herbal products. Adapted from **Table A3** (supplementary material, Appendix A)

			Extraction conditions (HPE)						Analyses					
Material	**Extracted compounds**	**Pre-treatment**	**Pressure (MPa)**	**Time (min)**	**Temperature (ºC)**	**Ratio mass to volume (g/mL)**	**Solvent**	**Other extraction methods**	**Antioxidant**	**Anti-tumoral**	**Anti-microbial**	**Structural changes**	**Other analyses (methods)**	**Reference**
American Ginseng	Ginsenosides	Dried in vacuum	200	2	25	1/50	60% Ethanol	US; MW; SC-CO_2; Soxhlet; Heat reflux	-	-	-	-	HPLC	Shouquin et al. (2006)
Rhodiola sachalinensis	Flavonoids; salidroside	Dried in vacuum	500	3	RT	1/70	60% Ethanol	US; Leaching; Soxhlet; Heat reflux	DPPH	-	-	-	HPLC	Zhang, Bi and Liu (2007)
Ginseng	Ginsenosides	Dried in oven	200	5	60	1/50	70% Ethanol	MW; US; Soxhlet; Heat reflux	DPPH	-	-	SEM	Colorimetry	Chen et al. (2009)
Green tea	Phenolic compounds	Dried in vacuum	500	1	RT	1/20	50% Ethanol	RT; US; Heat reflux	-	-	-	-	Phenolic compounds (Folin-Ciocalteu)	Xi et al. (2009)
Green tea	Caffeine	Dried in vacuum	500	1	RT	1/20	50% Ethanol	RT; US; Heat reflux	-	-	-	-	-	Xi (2009)
Schisandra chinensis	Deoxyschisandrin / *y*-schisandrin	Dried in vacuum	400	5	RT	1/90	90% Ethanol	Heat reflux; US	DPPH	-	-	-	Identification (HPLC)	Liu, Zhang and Wu (2009)
Berberis koreana	Phenolic compounds	Crude	500		RT	1/10	Water	US; Solid-liquid extraction	DPPH; Xanthine oxidase	Cell lines (A549, MCF-7, Hep3B, AGS, EK293); Human NK cell growth	-	-	HPLC; Phenolic compounds (Folin-Ciocalteu)	Qadir et al. (2009)
Korean barberry	Phenolic compounds	Dried stem brought	500	30	30	1/90	Distilled water	Solid-liquid extraction	-	Ames Salmonella mutagenicity	Probiotic activity; MIC	-	pH; Phenolic compounds (Folin-Ciocalteu)	Lee, He and Ahn (2010)
Deodeok roots	Phenolic compounds and Flavonoids	Dried in cabinet-type convective drier, and grinded	500	30	50	-	70% Ethanol	Solid-liquid extraction	DPPH; Ferric reducing power	Ames Salmonella mutagenicity	Probiotic activity; MIC and MBC	-	pH; Phenolic compounds (Folin-Ciocalteu); Identification (HPLC)	He et al. (2010)
Ginseng	Ginsenosides	Dry powder (purchased)	600	5	RT	-	Water	RT	-	-	-	-	Identification (HPLC)	Shin et al. (2010)
Green tea	Catechins and caffeine	Dried in vacuum	400	15	RT	1/20	50% Ethanol	Solid-liquid extraction	-	-	-	-	HPLC	Xi et al. (2010)

Table 1.3. (continued) High pressure assisted extraction studies in literature; focus on herbs and herbal products. Adapted from **Table A3** (supplementary material, Appendix A)

Ginseng	Ginsenosides	Fresh roots *versus* Dried roots	80	12 h	30	1/20	Water	Heat extraction	-	-	-	-	Ginsenosides analysis (HPLC); Phenolic compounds (Folin-Ciocalteu); Total sugars (phenol-H_2SO_4); Volatile compounds (GC-MS)	Lee et al. (2011)
Deodeok	Phenolic compounds; Flavonoids	Inlet air temperature	300	20	30	1/5	Water	Heat extraction	DPPH	Sulforho-damine B assay Cell line (HEK-293)	-	-	Phenolic compounds (Folin-Ciocalteu); Identification (HPLC)	He et al. (2011)
Green tea	Phenolic compounds	Fresh leaves pulverized	400	15	RT	1/20	50% Ethanol	-	-	-	-	SEM; TEM	-	Xi et al. (2011a)
Epimedium koreanum Nakai	Flavonoids	Dry powder (purchased)	350	5	-	-	50% Ethanol	Ultrasounds; Heat reflux; SC-CO_2	-	-	-	-	Total flavonoids (colorimetry)	Hou et al. (2011)
Green tea	Phenolic compounds	Dried in hot air oven	450	5	RT	1/20	50% Ethanol	Solid-liquid extraction	DPPH; Phospho-molybdenum	-	-	-	Phenolic compounds (Folin-Ciocalteu)	Xi et al. (2011b)
Dysosma versipellis	Podophyllotoxin; 4'-demethyl-podophyllotoxin	Crude	200	1	-	1/12	80% Methanol	Heat reflux	-	-	-	-	HPLC; ESI-MS; NMR	Zhu et al. (2012)
Green tea	Phenolic compounds	Dried in hot air oven	500	15	RT	1/20	50% Ethanol	-	-	-	-	-	Phenolic compounds (Folin-Ciocalteu)	Xi et al. (2013)
Moringa seeds	Essential oil	Purchased and cleaned	19.63	27.17	85.57	-	Water	-	-	-	-	-	Moisture content and yield	Fakayode and Ajav (2016)

Comparing HPE with other technologies, the authors reported that HPE allows obtaining the highest yield (7.70%) using a pressure level of 500 MPa for 2 min at room temperature, using 50% ethanol as solvent (ratio mass to solvent of 1:75), while supercritical CO_2, water-reflux, 50% ethanol-reflux, and 50% ethanol/ultrasonic extractions allowed to obtain yields of 2.32, 4.98, 5.75 and 5.89%, respectively **(Shouqin, Ruizhan and Changzheng, 2007)**. It was noteworthy that all the conventional techniques needed about 4 h to complete the extraction, while HPE only needed 2 min to achieve the highest yield, leading to an energy consumption remarkably lower.

The authors also verified (using an optimization software) that the predicted yield was of 7.76%, indicating the repeatability of HPE since the experimental yield was 7.70% **(Shouqin, Ruizhan and Changzheng, 2007)**. Similar results were obtained for ginsenosides extraction from American ginseng, where the obtained optimal conditions for HPE (200 MPa, 2 min, 25 ºC, using 60% ethanol as solvent) allowed to obtain a better extraction yield (0.821%) than the other methods, such as supercritical CO_2 extraction (yield of 0.324% after 4 h), Soxhlet (0.697%, 8 h), ultrasounds assisted extraction (0.716%, 40 min), heat reflux (0.761%, 6 h), and microwave assisted extraction (0.785%, 15 min) **(Shouquin et al., 2006)**. Caffeine was also extracted by HPE from green tea leaves, being the optimum conditions predicted as 500 MPa, at room temperature, for 1 min, using 50% ethanol as solvent **(Xi, 2009)**. The extraction yields observed were similar to all the extraction methods, being the main difference the extraction time: extraction at room temperature for 20 h, ultrasonic extraction for 90 min, heat reflux extraction for 45 min, and HPE that only took 1 min to be complete **(Xi, 2009)**. These results are important to demonstrate that HPE has clearly advantages over other extraction methodologies, especially the shorter extraction time and the higher yield obtained.

An interesting application of HPE is its combination with conventional techniques, and such as its use as pre-treatment for extraction of complex compounds, such as pectin, whose extraction involves a multiple-stage physicochemical technique (with hydrolysis, purification and isolation), being direct boiling (60-100 ºC) for 20-360 min the most common methodology **(Oliveira et al., 2016)**. **Guo et al. (2012)** defined the optimal conditions for HPE of pectin from orange peel as 500 MPa, 55 ºC, 10 min, and a ratio of 1/50 (g/mL) and reported a pectin extraction yield of 20.44%, which was higher than the one obtained by traditional heating at 82 ºC, for 1 h (15.47%). Recently, **Oliveira et al.**

(2016) reported the effect of HPE as pre-treatment for traditional heating extraction of pectin from passion fruit peel, and the obtained results indicated that while conventional heating allowed an extraction yield of only 7.40% after 57 min, the combined treatment allowed to double it to 14.34% after only 17 min. It is noteworthy that the pectin obtained by HPE presented the smallest particle diameters (8.96-11.02 μm) and the best emulsifying stability (100%) at pH 3-5 **(Guo et al., 2014)**. These results demonstrate that HPE can be used in order to facilitate pectin extraction, becoming an alternative for food industry to obtain pectin from natural sources **(Guo et al., 2012; Oliveira et al., 2016)**.

1.3.3.2. High pressure assisted extraction effect on biological activities

The first reported work concerning the antioxidant activity of extracts obtained by HPE was developed by **Sánchez-Moreno et al. (2004)**, who studied carotenoid content and antioxidant activity of tomato puree extracts obtained by HPE (50-400 MPa, 15 min, 25 ºC). These authors reported that a pressure level lower than 200 MPa led to a decreased lycopene extractability, while a pressure of 400 MPa, without the addition of any additives, led to the best extraction, with 14% more lycopene than untreated tomato puree. The same was observed for β-carotene (an increase of 20%), total carotenoids (increase of 10%), and vitamin A (increase of 24%) **(Sánchez-Moreno et al., 2004)**. These results were related to the antioxidant activity detected, since, for an aqueous extract, an extraction using pressures between 50 and 400 MPa without additives, led to an increase of the activity towards DPPH free radical, while sodium chloride and citric acid caused a decreased in antioxidant activity **(Sánchez-Moreno et al., 2004)**.

Xi (2006c) compared the antioxidant activity of flavonoids from propolis using HPE (at 500 MPa, 1 min, at room temperature, and 75% ethanol as solvent) and heat reflux extraction. The authors reported a higher antioxidant activity (~70%, DPPH assay) of the HPE extracts when compared to heat reflux extraction (~60%, DPPH assay). In addition to perform better than other extraction techniques, HPE also produced extracts with higher antioxidant activity than the synthetic ones, such as ascorbic acid **(Prasad et al., 2009b; Prasad et al., 2009e)** and butylated hydroxytoluene **(Prasad et al., 2010b)**. **Zhang, Bi and Liu (2007)** studied ethanolic extracts from *Rhodiola sachalinensis* obtained after HPE and reported an antioxidant activity of about 92% (DPPH assay). Also **Chen et al. (2009)** obtained extracts from ginseng with 55% and with 58% DPPH radical scavenging activity

after HPE and heat reflux extraction, respectively; the great advantage is that HPE only took 5 min to complete the extraction, while heat reflux took over 4 h.

The first study reporting the effect of HPE on the extraction of phenolic compounds with anticancer activity was developed by **Prasad et al. (2009a)** with longan fruit pericarp. The authors described a 1.5-fold increase on phenolic recovery by HPE (20.8 ± 1.6 mg/g), when compared to the conventional extraction used as control (14.6 ± 0.2 mg/g), which was reflected on a higher total antioxidant activity for HPE extracts **(Prasad et al., 2009a)**. Relatively to anticancer activity, the MTT [3-(4,5-dimethyl thiazole-2yl)-2,5-diphenyl tetrazolium bromide] assay was used in order to determine the extracts cytotoxicity, and reported that at 50 μg/mL, HPE extracts presented higher anticancer activity than cisplatin (synthetic chemotherapy drug) (37.6% and 34.2% of inhibition of SGC-7901 (human gastric carcinoma) cell line) **(Prasad et al., 2009a)**. **Qadir et al. (2009)** have combined HPE (500 MPa, 5 min, at room temperature, using water as solvent) with ultrasounds (40 kHz, 1 h), and reported the obtained *Berberis koreana* bark extracts had the highest extraction yield, the highest scavenging activity against DPPH and xanthine oxidase, and the highest anticancer activity against A549 and MCF-7 cell lines, when compared to the extracts obtained only with HPE.

Usually, the antioxidant activity is proportional to the concentration of total phenolic compounds on the extracts **(Prasad et al., 2009d; Xi, 2006c; Xi and Shouqin, 2007)**. **Casquete et al. (2015)** studied the effect of HPE on the antioxidant and antimicrobial activities of phenolic compounds present in extracts from various citrus peels. It was observed that the antioxidant activity (for ABTS and DPPH assays), increased with the concentration of phenolic compounds, being the highest values reported after HPE at 300 MPa, for 3 min **(Casquete et al., 2015; Casquete et al., 2014)**. Concerning the antimicrobial activity, the extracts were tested against 20 different bacteria (ten Gram-negative and ten Gram-positive). It was observed a higher antimicrobial activity for lemon extract (larger inhibition zone, using disc diffusion assay), and a most effective activity (different bacteria inhibited) was observed for mandarin extract **(Casquete et al., 2015)**.

1.3.3.3. High pressure assisted extraction effect on compounds profile

In longan fruit pericarp, three compounds were identified by HPLC as the major phenolic acids in this matrix: gallic acid, ellagic acid, and corilagin, ranging from 2.3 to 9.6

mg/g dw (the lowest amount was found on the extracts from conventional extraction, while the highest value was obtained after HPE (9.65 mg/g)) **(Prasad et al., 2009a; Prasad et al., 2010a; Prasad et al., 2010b; Prasad et al., 2009c)**. In litchi fruit pericarp extracts, two flavonoids (epicatechin and epicatechin gallate), were identified and quantified as the major compounds, while catechin and procyanidin B2 were identified as the minor compounds, being its extraction of about 7.8-fold higher after HPE (400 MPa, 30 min, 25 ºC, using ethanol:HCl (85:15, v:v)) when compared to the conventional extraction (30 min, 25 ºC) **(Prasad et al., 2009b)**. In green tea there is a great amount of phenolic compounds, such as caffeine and catechins, with proven biological activity. **Xi et al. (2010)** extracted phenolic compounds from dried green tea leaves using HPE, and through HPLC was able to identify and quantify the major components (caffeine, epigallocatechin gallate, epicatechin gallate, epigallo catechin, epicatechin, and gallic acid). The authors reported that the concentrations present in the final extract were greatly influenced by the pressure level (as pressure increased, the concentration of phenolic compounds increased as well), and that the extraction yields achieved with HPE (400 MPa pressure, for 15 min at room temperature) were similar to those of organic solvent extraction for over 2 h **(Xi et al., 2010)**. An interesting result obtained by **He et al. (2010)** with deodeok roots extracts was that after fermentation with different probiotic strains was observed a different total of peaks, indicating the production of new metabolites during fermentation, and its conservation after HPE, and also that some flavonoids, such as quercetin, rutin, and kaempferol can be degraded by bacterial growth. After HPE (600 MPa, for 5 min, at room temperature, using water as solvent) of total ginsenosides from *Korean Panax* ginseng, it was observed a clear increase for all the seven studied ginsenosides and an additional two unknown higher peaks, when compared to control (extraction at room temperature, for 24 h) **(Shin et al., 2010)**. Also in fresh ginseng, it was identified a total of 39 volatiles (three acids, two alcohols, four aldehydes, four ketones, one furan, one pyran, and twenty four terpenoids), being the most of them identified in the fresh ginseng extract after HPE, while only 29 were identified in the extract after heat assisted extraction **(Lee et al., 2011)**. These results propose that these compounds can be destroyed during thermal processing, and that HPE can produce extracts with a high level of various components **(Lee et al., 2011)**. Podophyllotoxin and its derivatives are a group of compounds present in *Dysosma versipellis*, a rare herb from China with important biological activity against a variety of

diseases **(Zhu et al., 2012)**. These bioactive compounds were extracted by HPE (200 MPa, 1 min, solid/liquid ratio of 1:12 (g/mL) and 80% methanol as solvent), and the authors reported that podophyllotoxin (extraction yield of 5.1 mg/g) and 4'-demethylpodophyllotoxin (21.9 mg/g) were obtained in one-step separation, and an extraction time 30-fold lower than for heat reflux traditional extraction **(Zhu et al., 2012)**.

1.4. Response surface methodology and process optimization

In order to improve the performance of a process and to take the maximum benefit from it, it is necessary to perform an optimization study to discover which conditions should be applied to produce the best response possible. Extraction processes are usually optimized by using the 'one-factor-at-a-time' approach, which consists on monitoring each factor individually, while the others remain constant. Nevertheless, this methodology is often expensive and very time-consuming, not being able to predict the optimal extraction conditions since it neglect the potential interactions between factors **(Lundstedt et al., 1998)**. For so, it was necessary to develop the concepts of experimental design and process optimization, and the so-called multivariate techniques, such as response surface methodology (RSM).

RSM is a commonly used technique for optimization of extraction process, since it is a collection of mathematical and statistical tools employed in the optimization of the process where response is influenced by a few independent factors. This statistical tool can predict the optimum experimental conditions in order to maximize/minimize several responses, while considering the interaction between independent variables (factors) and dependent variables (responses) **(Bezerra et al., 2008)**. The application of RSM as an optimization technique should follow several steps: (i) selection of the independent variables, (ii) selection of the experimental design and performing the experiments, (iii) mathematic–statistical treatment of the experimental data through the fitness of a polynomial function, (iv) evaluation of the model's fitness, (v) verification if it is necessary to perform additional experiments due to displacement in direction to the optimal region, and (vi) obtaining the optimum values for each variable and response **(Bezerra et al., 2008)**. For so, before using an RSM methodology it is necessary to choose an experimental design that will help to define which and how many experiments will be carried out.

1.4.1. Experimental design

A design of experiments (DOE) comprises a specific set of experimental work defined by a matrix composed by different level combinations for each factor. For so, the selection of independent variables (and its levels) is the first step to build an experimental design. The factors and their levels, *i.e.*, the values each factor will assume, must be chosen according to the major effects that each one can cause to a system; they are usually chosen through screening studies (using full or fractional two-level designs) or by a thorough analysis of literature.

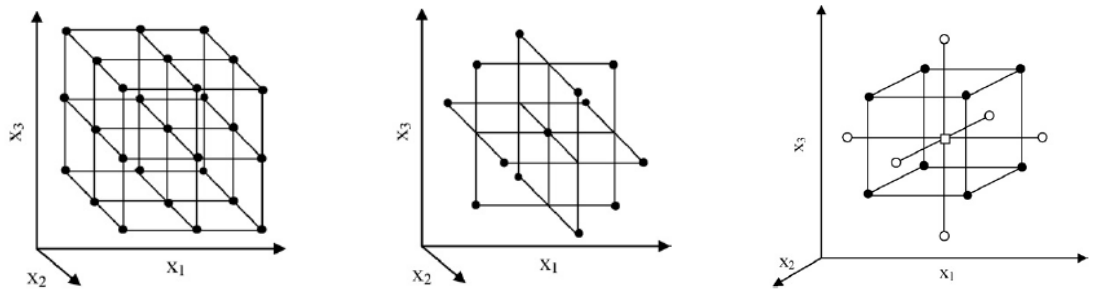
The simplest model which can be used in RSM is based in a linear function (**Table 1.4**), but it is presupposed that the responses cannot present any curvature; in these cases, a second-order model is used, with aid of determination of central-points which will help to define the curvature. The addition of central-points allows to minimize the risk of missing non-linear relationships in the middle of the intervals and their repetition allows the determination of confidence intervals. Furthermore, in order to determine critical points, such as maximum or minimum values, it is necessary to apply a polynomial function with quadratic terms, where each variable must have, at least, three levels **(Bezerra et al., 2008; Minitab, 2019)**. These equations are solved by using the statistical approach of the method of least square (MLS), which is responsible to fit the experimental data into a mathematical model, while generating the lowest residuals (difference between the predicted and experimental values) possible. After definition of which variables should be investigated, it is necessary to choose an experimental design. A factorial design enables the study of the influence of all experimental variables (*k*) and the interaction between them. To investigate the effects of *k* variables in a full factorial design, 2^k experiments are needed. Nevertheless, when *k* is higher than two, the number of possible experimental combinations required is very large (**Figure 1.6**), turning this method not appealing for modelling quadratic functions **(Bezerra et al., 2008; Lundstedt et al., 1998)**. When more than two factors are needed, experimental designs such as Box-Behnken and central composite arise.

Table 1.4. Different equations that can be used to obtain a response surface methodology

Linear model	$y = \beta 0 \sum_{i=1}^{k} \beta i x i + \varepsilon$
Second-order interaction model	$y = \beta 0 + \sum_{i=1}^{k} \beta i x i + \sum_{1<i<j}^{k} \beta i j x i x j + \varepsilon$
Quadratic model	$y = \beta 0 + \sum_{i=1}^{k} \beta i x i + \sum_{i=1}^{k} \beta i i x i^2 + \sum_{1<i<j}^{k} \beta i j x i x j + \varepsilon$

where k is the number of variables, β0 is the constant term, βi represents the coefficients of the linear parameters, ϰi represents the variables, βij represents the coefficients of the interaction parameters, βii represents the coefficients of the quadratic parameters, and ε is the residual associated to the experiments.

In Box-Behnken experimental design, the experimental points ate located on a hypersphere equidistant from the central point (**Figure 1.6**), and all factors should be adjusted at only three levels equally spaced between them **(Bezerra et al., 2008; Lundstedt et al., 1998)**.



Full factorial Box-Behnken Central composite

Figure 1.6. Experimental designs based on the study of three variable at three levels. Adapted from **Bezerra et al. (2008)**

These conditions allow to reduce significantly the number of experiments needed: a Box-Behnken design with three-variable-three-level, has only 13 experimental points, compared to the 3^3 = 27 experiments needed for a three-variable-three-level full factorial design. These designs can efficiently estimate the first and second-order coefficients but cannot include experiments from a factorial experiment, thus failing at predicting optimal conditions when the extreme conditions are all set at the same time **(Minitab, 2019)**.

A central composite design needs extra experimental points, at a distance α from its central point (named as axial points), besides the central point itself, allowing for the

factors to have until 5 levels each. These characteristics enable this model to fit a full quadratic model, contrarily to Box-Behnken designs which usually present fewer design points **(Minitab, 2019)**. A face-centred central composite design is a type of central composite design where α equals to 1; in this design the axial points are at the centre of each face of the factorial space (face of the 'cube'), and requires only three levels for each factor **(Minitab, 2019)**.

The major differences between Box-Behnken and a face-centred composite design is the fact that although both designs allow only three levels per factor, the Box-Behnken design does not comprise the points at the extreme conditions (*i.e.*, at the vertex of the cube, **Figure 1.6**), while the face-centred composite design includes all the extreme conditions even though the axial points are at the face of the cube. These characteristics make the face-centred composite design more predictive than Box-Behnken within the experimental domain.

1.5. General outlook

Bioactive components from plants are interesting due to its general preference over synthetic ones, and extraction is an essential step for obtaining them. There are several extraction methods, but most of them need to employ high temperatures and take long periods of time to be complete, which can lead to compound degradation and loss of its biological value. High pressure extraction is a new extraction technique with many advantages, being the main one that use low or room temperatures, avoiding thermal damage effects. Nevertheless, since there are several parameters to consider for obtaining an optimal process, a response surface methodology must be followed in order to optimize the extraction of bioactive compounds from stinging nettle and winter savory leaves. After extraction, chemical composition and several biological activities such as antioxidant activity, antimicrobial effect, as also their genotoxicity and cytotoxicity should be studied in order to observe the effect of high pressure assisted extraction in these parameters. It is expected that high pressure assisted extraction will require shorter extraction time, higher yields, and particularly the production of extracts with improved biological properties.

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CHAPTER II

OBJECTIVES AND WORK PLAN

Summary

2.1 Objectives

The aim of this work was to study high pressure assisted extraction (HPE) as a new extraction method to obtain herbal components from stinging nettle and winter savory leaves, avoiding the use of high temperatures. The few publications available on this technology (see **Chapter I**) demonstrate a great potential to develop a better alternative to the extraction methods in use, but further research on process optimization is required. The major objective was to establish the optimal pressure level, time of extraction, solvent composition, and $mass_{herb}$ to $volume_{solvent}$ ratio for herbal compounds extraction, which would provide higher extraction yield, higher extraction selectivity, and shorter extraction time, with no adverse side-effects on the activity of the bioactive components.

The work was performed into two major phases:

(I) optimization of the extraction process, where a wide range of pressures was studied at room temperature, as well as several pressurization times and different mass to volume ratios. Extraction yield, total polyphenols, total flavonoids, pigment content, as well as quantitative individual profile of phenolic compounds were studied for comparison purposes with control extraction;

(II) evaluation of the effect of HPE on the biological activities of the final extracts. For so, several biological properties were analysed, such as antimicrobial and anti-biofilm formation, antioxidant, anti-hypertensive, and pre-biotic activities, as also the genotoxicity, cytotoxicity, and anticancer properties of the final optimized extracts.

In order to do that, the work followed some specific objectives:

- Deep and systematic bibliographic research;
- Effect of HPE on herbs extraction yield and influence of pressure, time, solvent concentration and mass to volume ratio;
- Analysis of phenolic compounds, flavonoids and pigment contents, as also analysis of individual profiles;
- Effect of HPE on several biological activities of the optimized extracts;
- Evaluation of genotoxic and cytotoxic properties of extracts.

2.2 Work Plan

Figure 2.1. Thesis work outline.

CHAPTER III

MATERIALS AND METHODS

Summary

3.1. Chemical materials

2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid (Trolox), peptidil-dipeptidase A, EC 3.4.15.1, ECACC (ACE) (reference 09042001), deoxyribonucleic acid salt (DNA) from calf thymus, bromophenol blue sodium salt, phenazine methosulfate solution (PMS), fluorescein, potassium phosphate dibasic, 2,20-azo-bis-(2-methylpropionamidine)-dihydrochloride (AAPH), trypot-casein soy both (TSB), and peptone from animal tissue were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, EUA). Ethanol absolute anhydrous was obtained from Carlo Erba Reagents (Val-de-Reuil, France). Folin–Ciocalteu reagent, potassium acetate, and sodium carbonate anhydrous were purchased from AppliChem Panreac (Darmstadt, Germany). Potassium persulfate, rutin, aluminium trichloride extra pure anhydrous, and 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) were from Acros Organics (Geel, Belgium). Gallic acid hydrate was purchased from Tokyo Chemical Industry (Tokyo, Japan), methanol, glaciar acetic acid, and iron (III) chloride were acquired from Chem-Lab (Zedelgem, Belgium). o-aminobenzoylglycyl-p-nitophenylalanylproline (Abz-Gly-Phe(NO_2)-Pro) was acquired from Bachem Feinchemikalien (Bubendorf, Germany). Tris hydrochloride (Tris-HCl) buffer, and hydrochloric acid were purchased from Fluka (Bucharest, Romania). Sodium dihydrogen phosphate, sodium chloride, zinc chloride, hydrogen peroxide, 2-thiobarbituric acid, Ringer's solution, plate count agar, violet red bile dextrose agar, rose bengal chloramphenicol agar, and deMan, rogosa and sharpe agar were acquired from Merck (Darmstadt, Germany). Agarose and GreenSafe Premium were purchased from Nztech (Lisboa, Portugal). Iron(III) chloride ($FeCl_3$) and sodium acetate 3-hydrate were acquired from Panreac (Barcelona, Spain). Tris-Acetate EDTA buffer (TAE buffer) was purchased from Grisp (Porto, Portugal). Dulbecco's Modified Eagle's Medium (DMEM), Pen-Strep, and non-essential amino acids 100x were purchased from Lonza (Basel, Switzerland). Fetal Bovine Serum (FBS) was obtained from Biowest (Nuaillé, France). Tricholoacetic acid was purchased from Scharlau (Barcelona, Spain). Ammonium iron (II) sulphate and XTT (2,3-bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5- Carboxanilide) were purchased from Thermo Fisher Scientific (Porto Salvo, Oeiras, Portugal). Caucasian colon carcinoma (Caco-2), human colon adenocarcinoma cell line (HT29-MTX), human

squamous carcinoma (TR146), and human cervical carcinoma (HeLa) cell line cultures were obtained from the European Collection of Authenticated Cells Cultures (ECACC 8601020) through Sigma-Aldrich (St. Louis, USA); Human keratinocyte cell line (HaCat) was obtained from Cell Line Services (Appenheim, Denmark). *Escherichia coli* (ATCC 25922), methicillin-sensitive (MSSA) *Staphylococcus aureus* (MSSA ATCC 25923), and *Samonella enteriditits* (ATCC 13076), were obtained from American Type Culture Collection; *Bacillus cereus* (NCTC2599), *Listeria monocytogenes* (ESB 3562 (a food isolate from Escola Superior de Biotecnologia's culture collection, Porto, Portugal), *Lactobacillus acidophillus* LA-5, and *Lactobacillus casei* had their origin not discriminated.

Standard curves are given in **Appendix B**: **Table B1** presents the standard curves for total phenolic compounds, total flavonoids, and antioxidant activities assays; **Table B2** presents the standard curves used in LC-MS/MS analysis; and **Table B3** presents the standard curves used in HPLC analysis.

3.2. Biological samples

3.2.1. Herbal material

Stinging nettle (*Urtica dioica* L.) was harvested on May 2016, from Castro Daire region, Portugal. A batch of 4 kg was collected, transported under refrigeration, and washed with tap water. After removal of excess water, nettle was separated in leaves, stems, and roots (**Figure 3.1**), grounded and then packed under vacuum and stored at -45 ºC until further analyses. For the present work, only the leaves were used. Winter savory (*Satureja montana* L.) dried leaves were offered by Cantinho das Aromáticas (Vila Nova de Gaia, Portugal), and were harvested in October 2018.

Stinging nettle Winter savory

Figure 3.1. Initial samples for stinging nettle and winter savory

3.2.2. Preparation of carrot juice

Fresh carrots (*Daucus carota* subsp. sativus) were purchased at a local store (Aveiro, Portugal) and washed with tap water to remove dust and other adhered particles. After being cut in small pieces, the carrots were blended with distilled water (150 g per 300 mL of water) and filtered with a cotton filter to remove pulp **(Pinto et al., 2018) (Figure 3.2)**.

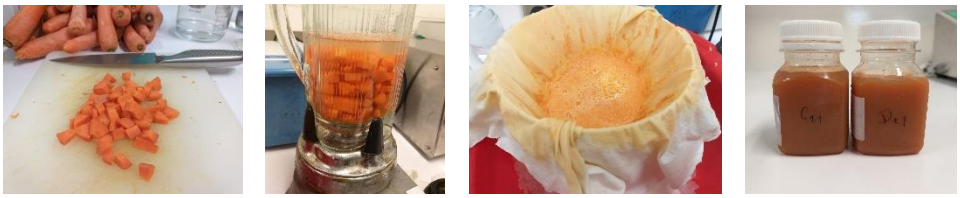


Figure 3.2. Preparation of carrot juice

Immediately after preparation, juice was separated into aliquots and transferred to sterilised flasks where the extract in a final concentration of 1 mg/mL was added (see **Table 3.1** for nomenclature). All juices were stored under refrigeration.

Table 3.1. Identification of samples (nomenclature)

	Nomenclature	Conditions		
		Pressure level	**Extraction time**	**Solvent**
Chapter V (article 4)	**N200/10.6/0**	200 MPa	10.6 min	0% Ethanol
	N0.1/10.6/0	0.1 MPa	10.6 min	0% Ethanol
	N500/10/25	500 MPa	10.0 min	25% Ethanol
	N0.1/10/25	0.1 MPa	10.0 min	25% Ethanol
	N200/10.6/35	200 MPa	10.6 min	35% Ethanol
	N0.1/10.6/35	0.1 MPa	10.6 min	35% Ethanol
	N200/10.6/70	200 MPa	10.6 min	70% Ethanol
	N0.1/10.6/70	0.1 MPa	10.6 min	70% Ethanol
Chapter V (article 5)	**S500/20/0**	500 MPa	20 min	0% Ethanol
	S0.1/20/0	0.1 MPa	20 min	0% Ethanol
	S348/20/35	348 MPa	20 min	35% Ethanol
	S0.1/20/35	0.1 MPa	20 min	35% Ethanol
	S500/1/70	500 MPa	1 min	70% Ethanol
	S0.1/1/70	0.1 MPa	1 min	70% Ethanol
Chapter VI (article 6)	**Juice**	Control carrot juice		
	Juice + HPP	Control carrot juice pasteurized by high pressure		
	Juice + Extract	Carrot juice with addition of extract		
	Juice + Extract + HPP	Carrot juice with addition of extract pasteurized by high pressure		

3.3. Extraction conditions

High pressure extraction (HPE) was carried out at room temperature (20-25 ºC), and according to the experimental design different extraction times (1, 10.5, and 20 min), different solvents (0% ethanol (aqueous), 35% ethanol, and 70 % ethanol), and different pressure levels (200, 350, and 500 MPa) were studied. Initially, the nettle extracts were obtained by placing 2.00 g of plant material in a container together with 20 mL of extraction solvent and the models construction was performed using this mass/volume ratio (1/10). Nevertheless, after performing an additional ratio study (**Appendix C1**), the mass/volume ratio of 1/20 was used in the optimized extracts (to who's the biological activities where studied). For the winter savory samples, the ratio study (**Appendix C2**) was performed before the optimization process and the several response models, as well as the biological activities studied, were performed using the mass/volume ratio of 1/10.

Samples were homogenized with an Ultraturrax T25 homogeniser (Janke & Kunkel IKA-Labortechnik) (**Appendix C3**), and the mixture was then placed in low permeability polyamide–polyethylene bags (PA/PE-90, Albipack, Packaging Solutions, Águeda, Portugal) that were heat sealed manually with care to avoid as much as possible to leave air inside the bags. HPE experiments were carried out on an industrial scale high pressure equipment (Model 55, Hiperbaric, Burgos, Spain) with a pressure vessel of 55 litters, a 200 mm diameter, and 2000 mm length with a maximum operation pressure of 600 MPa. Control samples were maintained at atmospheric pressure (0.1 MPa) under the same conditions of temperature, time, and solvent concentration. Controls were kept in the dark and surrounded by water to mimic all the conditions of the samples under pressure, except for the high pressure (see **Table 3.1** for nomenclature).

After extraction, each sample was centrifuged at 15000 rpm, 10 min, at 4 ºC (Heraeus Biofuge Stratos, Thermo, Electron Corporation, Massachusetts, EUA). The supernatant was then filtered using a 10-13 µm filter (Whatman nº 1 equivalent, 1250 Filter-Lab, Filtros Anoia, S.A., Barcelona, Spain), and the filtrates were collected and stored at -80 ºC until further analyses. All extractions were performed in triplicate.

3.4. Response surface methodology

A response surface methodology (RSM) (**Appendix D**) was used to analyse the potential relationships between the independent variables (individual effects and possible

interactions), as well as to find the optimum extraction conditions for each studied response. The experimental design followed a central composite face-centred design (CCD) using Minitab Statistical Software v.17.0 (Minitab Ltd., Coventry, United Kingdom). Previous studies on herbal compounds extraction by high pressure found in literature served as basis for the screening of the factors and corresponding levels; three factors were tested (pressure level (X_1), holding time (X_2), and solvent concentration (X_3)) at three levels i.e., lower (-1), intermediate (0), and higher (+1) (see **Table 3.2**), in a total number of 14 random experimental runs and 6 replicates at the central point (for error assessment).

Table 3.2. Real and coded values of independent factors studied by response surface methodology

Factors	Coded variable levels		
	-1	**0**	**+1**
X_1: Pressure level (MPa)	200	350	500
X_2: Time of extraction (min)	1	10.5	20
X_3: Solvent concentration (%)	0	35	70

Response variables were defined as extraction yield, total phenolics, total flavonoids, chlorophylls, carotenoids, and *in vitro* antioxidant activity (ABTS and DPPH scavenging activities, and ferric reducing antioxidant activity). All responses were estimated in triplicates of samples and triplicates of analyses. The CCD follows a second order polynomial as in equation 3.1 (Eq. 3.1):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta iXi + \sum_{i=1}^{k} \beta iiXi^2 + \sum_{i=1}^{k} \sum_{j=i+1}^{k-1} \beta ijXiXj \qquad \text{(Eq. 3.1)}$$

where, Y is the response (dependent variable), X_i and X_j are the independent variables, and k is the number of tested variables (k = 3). Model coefficients are defined as β_0 for intercept, β_i for linear terms, β_{ii} for quadratic terms, and β_{ij} for interaction terms. Regression coefficient value (β) was estimated to investigate positive or negative effects of individual factors over the response and was further used to generate three-dimensional response surface graphs for individual responses.

The not statistically significant terms ($p>0.15$) were dropped to simplify the models by a stepwise procedure. Analysis of variance (ANOVA) was carried out to determine F-value

for the significant ($p<0.05$) linear, quadratic, and interaction effects. Lack-of-fitness was also determined for each response model and fitness of polynomial equation was estimated using the coefficient of determination (R^2). A simple procedure was used to optimize the different variables to a maximum response through each mathematical model using Minitab Statistical Software v.17.0. This method allowed to predict the optimal conditions for each extraction, and the consequent maximum value (**Appendix C6**). After each optimal condition set performance, the obtained experimental values were compared to the predicted ones and the difference between the two values was accessed by percentage.

3.5. High pressure treatment of carrot juice

Juice aliquots (30 mL) were placed in low permeability polyamide-polyethene bags. The bags were manually heat sealed and placed in a cylindrical loading container at room temperature to be pressurized at 550 MPa for 3 min **(Koutchma, Popović, Ros-Polski, & Popielarz, 2016)**. High pressure pasteurization was carried out in the same equipment used for HPE. All samples were performed in triplicate and stored at 4 °C in the dark up to 15 days for the storage study. For each sample, several bags were prepared so that three new bags could be opened aseptically at each sampling time (0, 5, and 15 days of storage).

3.6. Extraction yields

The extraction yield was calculated according to the method described by **Zhang, Bi and Liu (2007)**. Briefly, the extracts were evaporated by a rotary evaporator (Rotavapor R-210, Büchi Labortechnik, Flawil, Switzerland) under vacuum (Vacuum Pump V-700, Büchi Labortechnik, Flawil, Switzerland) at 40 ºC, and lyophilized in a freeze-dryer (VirTis Benchtop K, SP Scientific, NY, USA). The extraction yields were calculated per gram of dry basis material (DM) according to the equation 3.2.

$$\text{Yield (\%)} = (m/m_{residue}) \times 100;\ m_{residue} = (m_{initial} / V_{filtrate}) \times V_{evap} \quad \text{(Eq. 3.2)}$$

where m is the mass of the dried extract, $m_{residue}$ is the mass of the initial extract; $m_{initial}$ is the mass of herb used for the extraction, $V_{filtrate}$ is the volume of extract obtained after filtration, and V_{evap} is the volume of extract used for evaporation (1 mL).

3.7. Quantification of total phenolics

Total phenolic compounds from extracts were quantified by the Folin-Ciocalteu method **(Singleton, Orthofer and Lamuela-Raventós, 1999)**. Folin-Ciocalteu reagent

(100 µL) was mixed with 20 µL of extract in a microplate with 96 wells. After resting for 4 min, 75 µL of sodium carbonate solution (100 g/L) were added and the mixture rested for 2 h in the dark, at room temperature. The absorbance at 750 nm was registered (Multiskan Go microplate spectrophotometer, Thermo Fisher Scientific Inc., USA). Gallic acid was used as standard (0–200 mg/L) (calibration curve in **Table B1, Appendix B**) and the results were expressed as milligram of gallic acid equivalents per gram of dry matter (mg_{GAE}/g DM).

3.8. Quantification of total flavonoids

The total flavonoid content was determined according to the method described by **Chang et al. (2002)**. The reaction mixture consisted of 100 µL of 2% aluminium trichloride, 100 µL of potassium acetate, 1.5 mL of methanol, 2.8 mL of water and 500 µL of each extract. The mixture was allowed to react for 30 min at room temperature, in the dark. The absorbance was then read at 415 nm (Multiskan Go microplate spectrophotometer, Thermo Fisher Scientific Inc., USA). Standard solutions of rutin were prepared at different concentrations (0–500 mg/L) to obtain the calibration curve (**Table B1, Appendix B**) and the results were expressed as milligram of rutin equivalents per gram of dry matter (mg_{rutin}/g DM).

3.9. Quantification of chlorophylls and carotenoids

The concentration of individual and total chlorophylls, as well as the concentration of carotenoids in nettle extracts was determined using the method of **Lichtenthaler (1987)**. The absorbance of the extracts was measured at 470, 649, and 664 nm in order to apply the equations 3.3 to 3.6:

$$\text{Chlorophyll a (ChlA)} = 13.36 \times Abs_{664} - 5.19 \times Abs_{649} \quad \text{(Eq. 3.3)}$$

$$\text{Chlorophyll b (ChlB)} = 27.43 \times Abs_{649} - 8.12 \times Abs_{664} \quad \text{(Eq. 3.4)}$$

$$\text{Total chlorophyll (Chl total)} = 5.24 \times Abs_{664} + 22.24 \times Abs_{649} \quad \text{(Eq. 3.5)}$$

$$\text{Carotenoids} = \frac{1000\ x\ Abs470 - 2.13\ x\ ChlA - 97.64\ x\ ChlB}{209} \quad \text{(Eq. 3.6)}$$

Where ChlA and ChlB are the concentrations of the individual chlorophylls, Chl total is the concentration of all chlorophylls present in the extract, and Abs_{470}, Abs_{649}, and Abs_{664}

are the absorbances at 470, 649, and 664 nm, respectively. All the results are expressed in microgram per milligram of dry matter (µg/mg DM).

3.10. Antioxidant activity

3.10.1. Radical cation ABTS scavenging activity

The radical scavenging ability of the extracts for the ABTS radical cation was performed according to the methodology described by **Re et al. (1999)**. To prepare the $ABTS^{\bullet+}$ solution, 7 mM of ABTS and 2.45 mM potassium persulfate reacted (1:1, v/v) for at least 16 h in the dark at room temperature. Then the $ABTS^{\bullet+}$ radical was diluted to an absorbance of 0.70 ± 0.02 at 734 nm (Multiskan Go microplate spectrophotometer, Thermo Fisher Scientific Inc., USA); the dilutions were performed with the solvent of each extract, *i.e.*, water, 35% ethanol, and 70% ethanol. Each radical solution (200 µL) was then allowed to react with 20 µL of each extract for 6 min, and the optical density was measured at 734 nm using the same microplate reader described above. The standard curve was linear between 0 and 600 µM Trolox (**Table B1, Appendix B**). The extracts antioxidant activity was expressed as milligrams of Trolox equivalents per gram of dry matter (mg_{trolox}/g DM).

3.10.2. Radical cation DPPH scavenging activity

The methodology followed was based on the assay described by **Bobo-Garcia et al. (2015)**. Briefly, 20 µL of each extract were added to a 96-well plate containing 180 µL of 150 µM DPPH solution. The mixture was allowed to react for 40 min, in the dark, at room temperature, and the optical density was measured at 515 nm using the same microplate reader described above. The standard curve was linear between 50 and 500 µM Trolox (**Table B1, Appendix B**). The percentage of inhibition was determined, and the extracts antioxidant activity was expressed as milligrams Trolox equivalents per gram of dry matter (mg_{trolox}/g DM).

3.10.3. Ferric reduction antioxidant power

The ferric reducing antioxidant power (FRAP) assay was performed in a microplate, according to **Bolanos de la Torre et al. (2015)**. The FRAP working solution was prepared daily and warmed at 37 ºC before use; it consisted on mixing 300 mM acetate buffer (pH

3.6), 40 mM 2,6-tripyridyl-s-triazine (TPTZ) dissolved in 40 mM hydrochloric acid, and 20 mM ferric chloride (10:1:1, v/v/v). Each sample (20 µL) was then allowed to react with 200 µL of FRAP reagent for 30 min, at 37 ºC, in the dark. The absorbance was measured at 593 nm. The standard curve was linear between 0 and 500 µg/mL ammonium iron (II) sulfate (AIS) (**Table B1, Appendix B**). The extracts antioxidant activity was expressed as milligrams AIS equivalents per gram of dry matter (mg_{AIS}/g).

3.10.4. Oxygen radical absorbance capacity

The oxygen radical absorbance capacity (ORAC) assay was performed **(Amorim et al., 2018)**. Briefly, the reaction was carried out at 40 ºC in black polystyrene 96-well microplates (Nunc, Denmark) using 75 mM phosphate buffer (pH 7.4); the final assay mixture contained 120 µL fluorescein (116.7 nM), 60 µL AAPH (48 nM), and 20 µL of antioxidant (Trolox [10 at 80 mM] or sample [at different concentrations]). The fluorescence was recorded during 97 min in a FluoSTAR OPTIMA microplate reader (BMG Labtech, Offenburg, Germany), with 485 nm excitation and 520 nm emission filters. The equipment was controlled by the FluoSTAR Control software version 1.32 R2 for fluorescence measurement. AAPH and Trolox solutions were prepared daily and fluorescein was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer (pH 7.4). All reaction mixtures were prepared in duplicate and at least three independent runs were performed for each sample. ORAC results were expressed as mg Trolox/g dry matter (mg_{trolox}/g) (calibration curve in **Table B1, Appendix B**).

3.11. Analysis of phenolic acids by HPLC

Individual polyphenols analysis was carried out according to the method proposed by **Oliveira et al. (2015)**. Qualitative profiles of polyphenols were conducted on a Waters 2685 Separations Module (Waters Corporation, Milford, MA, USA). The PDA acquisition wavelength was set in the range of 200–600 nm, being the analog output channel A at wavelength 280 nm and the analog output channel B at 320 nm. The solvent gradient varied from mixture solvent A (0.2:5:94.8, TFA:acetonitrile:water) to mixture solvent B (0.2:99.8, TFA:acetonitrile), with a flow rate of 1 mL/min. The mobile phase composition started at 100% solvent A for 1 min, followed by a linear increase of solvent B to 21% in 30 min, till a maximum of solvent B at 58% after 55 min, and then bring mobile phase

composition back to the initial conditions after 60 min for the next run. The mobile phase was degassed before injection onto HPLC. The injection volume of each sample was 40 µL. Phenolics were identified and quantified by comparison with commercially standards and respective calibration curves (**Table B2, Appendix B**).

3.12. Analysis of phenolic acids by LC-MS/MS

Phenolics identification/quantification was conducted on a UPLC Ultimate 3000, Dionex liquid chromatograph coupled to an UHR-QqTOF (Ultra-High Resolution Qq-Time-Of-Flight) mass spectrometry (Impact II™, Bruker, Massachusetts, EUA). Mobile Phase: Solvent A: ultra-pure water (100%) (Millipore system) with 0.1% formic acid (Sigma-Aldrich, Germany), and Solvent B: acetonitrile (100%) (Merck pure grade) with 0.1% formic acid; at a flow rate of 0.25 mL/min. The following gradient was employed: 0-10 min (0% B); 10-14 min (21% B); 14-18.3 min (27% B); 18.3-20 min (58% B); 20-21.5 min (95% B) and 21.5-22 min (0% B). Each run took 21 min to complete. The capillary voltage of the electrospray ionization (ESI) was set to 2500 V and the capillary temperature was 200 °C. Spectra were recorded in negative-ion mode between m/z 20 and 1000. Phenolics were identified and quantified by comparison with commercially available standards and respective calibration curves (**Table B3, Appendix B**). Results were expressed as milligram per 100 grams of dry matter (mg/100 g DM).

3.13. Determination of antihypertensive activity

The antihypertensive activity was determined by the angiotensin-I converting enzyme (ACE) inhibition assay with some modifications **(Sentandreu and Toldra, 2006).** To each black polystyrene 96-well microplates (Nunc, Denmark), 160 µL of fluorescent substrate Abz-Gly-Phe(NO2)-Pro in Tris-HCl buffer and 40 µL of each extract with different concentrations were added. The enzyme reaction was initiated by the addition of 2 mU of ACE (peptidil-dipeptidase A, EC 3.4.15.1), dissolved in glycerol (50%) and prepared in Tris-HCl buffer solution (150 mM) with 0.1 mM of ZnCl2, pH 8.3 that were immediately mixed and incubated at 37 ºC. The generated fluorescence was measured after 30 min by a multiscan microplate fluorimeter, using the software FluoSTAR Control version 1.32 R2. The excitation and emission wavelengths were 350 and 420 nm, respectively. The inhibition of ACE activity was calculated using the Equation (3.7):

$$ACE\ activity\ (\%) = \frac{(Fcontrol - Fblank) - (Fsample - F\ sample\ blank)}{F\ control - F\ blank}\ x\ 100 \qquad \text{Eq. (3.7)}$$

Where $F_{control}$ is the fluorescence emitted by the o-aminobenzoylglycyl complex through the activity of ACE on the fluorescent substract Abz-Gly-Phe(NO2)-Pro; F_{blank} is the fluorescence emitted by the fluorescent substract Abz-Gly-Phe(NO2)-Pro; F_{sample} is the fluorescence emitted by the o-aminobenzoylglycyl complex through the activity of ACE on the fluorescent substract Abz-Gly-Phe(NO2)-Pro in the presence of a possible inhibitor; and $F_{sample\ blank}$ is the fluorescence emitted by the fluorescent substract Abz-Gly-Phe(NO2)-Pro in the presence of a possible inhibitor. The extracts antihypertensive activity was expressed as percentage of inhibition of angiotensin-I converting enzyme (%).

3.14. Determination of antimicrobial and antibiofilm activities

In this work *Escherichia coli* (ATCC 25922), methicillin-sensitive (MSSA) *Staphylococcus aureus* (MSSA ATCC 25923), *Salmonella enteritidis* (ATCC 13076), *Bacillus cereus* (NCTC2599), and *Listeria monocytogenes* were used as target pathogens. An inoculum of each bacteria was prepared from overnight cultures and inoculated in TSB (trypto-casein soy broth). Each lyophilized extract was re-suspended and mixed with TSB and filtered through a 0.22 µm filter to ensure that no contamination occurred.

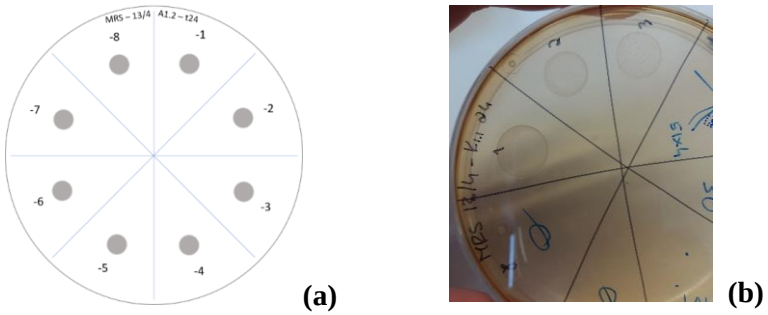
Minimum inhibitory and bactericidal concentrations (MIC and MBC) were determined following the Clinical and Laboratory Standards Institute guidelines **(M07-A8, 2009; Silva et al., 2013).** Two test solutions for each extract, at 10 and 20 mg/mL, were prepared and inoculated at 1% (v/v) with an inoculum of 10^8 CFU/mL and incubated for 24 h at 37 ºC. The MIC was determined by observing the lowest concentration of extract that visually inhibited bacterial growth. The MBC was determined as the lowest concentration of each extract at which bacterial growth was prevented, and the initial viability was reduced by at least 99.9% within 24 h **(Costa et al., 2012)**; For so, MBC was determined by inoculation on plate count agar of 20 µL aliquots of the mixtures that presented no turbidity in previous MIC determination, using the drop plate technique. All assays were performed in duplicate.

The antibiofilm activity was studied according to **Silva et al. (2016)** briefly consisted on a mixture, on a 96 well microplate (Nunc, Darmstadt, Germany), of each extract with TSB at 10 and 20 mg/mL (the extracts were filtered through a 0.22 µm filter to ensure that

were not contaminated) and each microorganism. The incubation was left to occur for 24 h, at 37 ºC, and then the contents of the plate were discarded, being each well carefully washed to remove the non-adhered cells and the biofilms were stained using crystal violet. After shaking the microplate for 15 min, at 320 rpm, the absorbance was read at 660 nm. All assays were done in triplicate, a positive control was drawn using inoculated culture media and a negative control was prepared using only sterile media.

3.15. Determination of prebiotic potential

Two bacterial strains (*Lactobacillus acidophillus* LA-5 and *Lactobacillus casei*) were studied for 24 and 48 h. An inoculum of each bacteria was prepared from overnight cultures and inoculated in MRS broth. Each lyophilized extract was re-suspended and mixed with base medium (to a final concentration of 2 mg/mL) and filtered through a 0.22 µm filter to ensure that no contamination occurred. A negative control was performed by mixture of each microorganism in base medium and the positive control consisted on FOS 2% (fruto-oligossacharide solution in base medium). Decimal dilutions (to a final volume of 1.0 mL) were prepared in peptone water for inoculation of each extract with a microorganism. Microbial growth was determined by inoculation of 20 µL aliquots of each dilution on MRS agar, using the drop plate technique (**Figure 3.3**).



(a) **(b)**

Figure 3.3. Drop seed method: (a) scheme-example of each Petri dish; (b) real example

3.16. Determination of extracts genotoxicity by DNA assay

The genotoxicity of the extracts was determinate by the DNA assay, using electrophoresis to access the level of denaturation of DNA when in the presence of the extract **(Silva et al., 2017)**.

3.16.1. DNA protection assessment (antioxidant assays)

The DNA solution (0.25 mg/mL) was incubated in the presence of the two degradation systems selected: (i) hydrogen peroxide (H_2O_2) 50 %, v/v and (ii) H_2O_2 50 %, v/v with $FeCl_3$ 10 mM and varying concentrations of extracts, with a range of volumes (400, 300, 200 and 100 µL), in PBS buffer. For so, the mixture of 400 µL H_2O_2 with y µL of sample, 400-y µL PBS and 200 µL DNA, to a final volume of 1000 µL was done (when studying the $FeCl_3$ system, it were added 10 µL of $FeCl_3$ 10 mM, and these volume was discounted on PBS volume). The DNA solution without H_2O_2 was used as a positive control (no degradation) for the assays using the H_2O_2 system, and a DNA solution with just PBS was used as a positive control (no degradation) for the assays using the H_2O_2/$FeCl_3$ system. The mixture was made in duplicate for each extract. After 1 h incubation at 37 ºC, in the dark, an agarose gel electrophoresis was run (**Figure 3.4**).

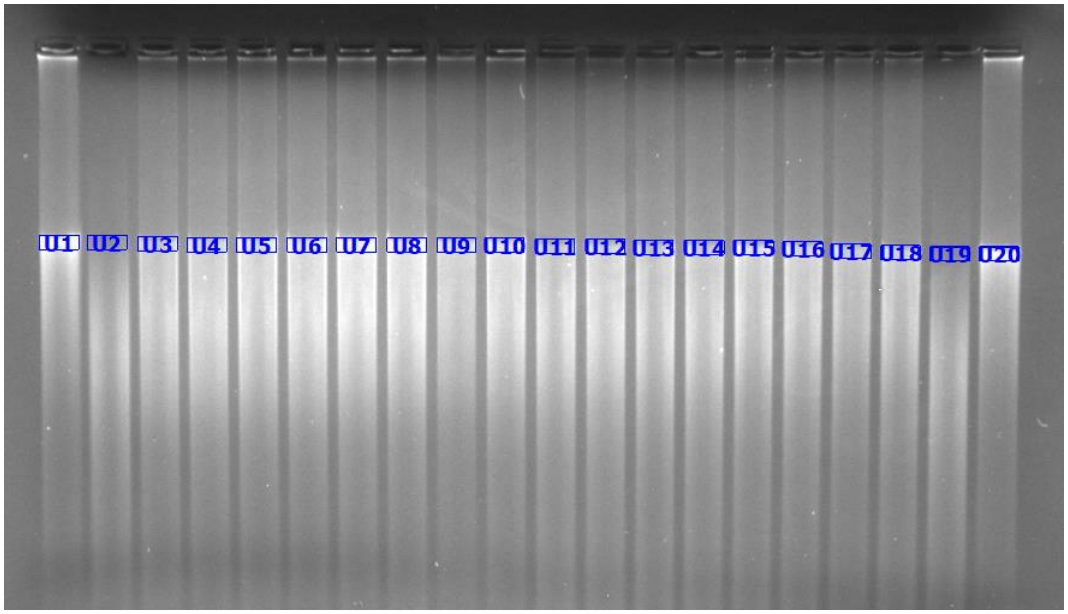


Figure 3.4. Schematic representation of the processing of the electrophoretic results after the integration of each band. U1 and U20 represent the DNA solution (positive control); U2 and U19 represent the DNA solution and degradation system (negative control); U3 to U18 represent the DNA solution mixed with the degradation system and with addition of the extract in different concentrations

3.16.2. DNA degradation assessment (pro-oxidant assays)

The DNA solution (0.25 mg/mL) was incubated in the presence/absence of $FeCl_3$ 10 mM and varying concentrations of extracts, with a range of volumes (400, 300, 200 and 100 µL), in PBS buffer. For so, the mixture of y uL of sample, 800-y uL PBS, and 200 uL

DNA, to a final volume of 1000 uL was done (when studying the $FeCl_3$ system, it were added 10 µL of $FeCl_3$ 10 mM, and these volume was discounted on PBS volume). The DNA solution without 10 mM $FeCl_3$ was used as a positive control (no degradation) for the assays using the $FeCl_3$ system. The mixture was made in duplicate for each extract. After 1 h incubation at 37 ºC, in the dark, an agarose gel electrophoresis was run (**Figure 3.4**).

3.16.3. Electrophoresis

Each sample was mixed 1:4 with loading buffer (25 mg bromophenol blue, 10 mL Tris EDTA (TE) buffer 1x pH 8.0, and 20 mL glycerol with pH value adjusted to 8.0) and 10 mL aliquots were transferred into a 0.75% (w/v) agarose gel prepared using Tris-Acetate EDTA buffer (TAE) supplemented with 0.03 mL/mL GreenSafe Premium. Electrophoresis was run for 1.25 h at 150 mV. Gels were analysed using a molecular imager GelDOC XR+ (BioRad, Hercules, California, USA) and the resulting image was processed using Image Lab Software v5.1 (BioRad, Hercules, California, USA). The band area for each positive control was manually defined (band intensity) and then copied into each sample lane (maintaining the distance to the wells; with the decrease in band intensity being considered as a result of a reduction of the amount of DNA present. The results were given as the percentage of inhibition of the DNA band degradation (for the antioxidant assay) (see equation (3.8)) or as percentage of DNA band degradation (for the pro-oxidant assay) (see equation (3.9))

$$Inhibition\ of\ DNA\ degradation\ (\%) = \frac{Intensity\ (Sample)}{Intensity\ (DNA\ solution)}\ x\ 100 \qquad \text{Eq. (3.8)}$$

$$DNA\ degradation\ (\%) = 100 - \frac{Intensity\ (Sample)}{Intensity\ (DNA\ solution)}\ x\ 100 \qquad \text{Eq. (3.9)}$$

Where *Intensity (sample)* is the intensity of each sample band, and *Intensity (DNA solution)* refers to the intensity of the intact DNA solution.

3.17. Determination of extracts cytotoxicity and anticancer activity

3.17.1. Cytotoxicity

Cell culture

Human colon carcinoma (Caco-2) cells were obtained from the European Collection of Authenticated Cells Cultures (ECACC 8601020 and were grown using high glucose (4.5

g/L) DMEM supplemented with 10% (v/v) heat inactivated FBS, 1% (v/v) Penicillin-Streptomycin-Fungizone (Lonza, Verviers, Belgium), and 1% (v/v) of non-essential amino acids 100x (Sigma, Germany). Human keratinocyte cell line (HaCat) was obtained from Cell Line Services (Appenheim, Denmark) and were cultivated in DMEM with 4.5 g/L glucose, L-glutamine without pyruvate containing 10% (v/v) FBS and 1% (v/v) Penicillin-Streptomycin-Fungizone (Lonza, Verviers, Belgium). All cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Caco-2 cells were assayed between passages 50 and 52 and HaCat cells were assayed between passages 77 and 80.

Cytotoxicity assay

Produced extracts cytotoxic potential was assessed upon the cell lines using the XTT colorimetric assay in accordance with ISSO 10993-5. Briefly, cells were seeded at a 1 x 10^5 cells/mL in the wells of a 96 well microplate and allowed to adhere. After 24h, the media was removed, the cells were washed with PBS. Following this media with extracts at various concentrations (1.0 and 5.0 mg/mL for stinging nettle and 0.5 and 1.0 mg/mL for winter savory) was added. After 24h, 25 µL of XTT were added to each well and the cells were incubated, in the dark, for 2 h. The optical density (OD) at 485 nm was then measured using a microplate reader (FLUOstar, OPTIMA, BMG Labtech, Ortenberg, Germany). The impact of extracts upon the cellular metabolism was quantified accordingly to the following formula (Equation (5)):

$$Metabolism\ inhibition\ (\%) = 100 - \frac{Optical\ density\ (Sample)}{Optical\ density\ (Growth\ control)}\ x\ 100 \qquad \text{Eq. (5)}$$

All extracts were assessed through two individual experiments executed within a week of each other. In each individual experiment all conditions were assayed in quintuplicate.

3.17.2. Citotoxicity and anticancer potential

Caco-2, human squamous carcinoma (TR146) and human cervical carcinoma (HeLa) cell line cultures were maintained in 75 cm^2 T-flasks (T-75) with DMEM and incubated in a 5% CO_2/95% air and 98% relative humidity atmosphere. When 70-80% of cells were confluent, the culture medium was removed, and the cells were rinsed with pre-warmed

PBS. Cells were then detached using trypsin-EDTA, at 5% CO_2 air atmosphere, diluted to the desirable cell density ($1x10^4$ cells per well), and left to incubate for 24 h (for HeLa and TR146 cell lines) and 48 h (for Caco-2 cell line) in a cell incubator at 37 ºC in a 5% CO_2 air atmosphere. The IC_{50} values (concentration of each extract needed to inhibit the cell metabolism in at least 50%) were calculated for TR146 cell line at passage 20-23, for HeLa cell line at passage 9-14, and for Caco-2 cell line at passage 28-33. The extracts were prepared in a working solution of 200 mg/mL in DMSO and diluted for 0.0002-2.0 mg/mL in DMEM. The negative control was prepared with 1% Triton X-100 in DMEM; the positive control was prepared with DMEM. 200 µL of each extract were added to the wells and incubation occurred for 24 h at 37 ºC in a 5% CO_2 air atmosphere. Then the supernatant was removed, and 200 µL of MTT solution (0.5 mg/mL) was added to each well and incubated for 4 h at 37 ºC. After that the MTT solution was discarded and replaced by 200 µL of DMSO. The mixture was shaken for 20 min at room temperature, 100-150 rpm, and the absorbance was read at 570 nm and 630 nm using a microplate spectrophotometer.

3.18. Case-study analyses

3.18.1. Microbiological analyses

After each period of storage (initial, 5, and 15 days), 1.0 mL aliquots were obtained aseptically and homogenised with 9.0 mL of Ringer's solution. From the original sample (dilution 10^0), decimal dilutions were prepared (up to a dilution of 10^{-4} allowing a maximum microbiological quantification of 6.0 $\log_{10}$ CFU/mL). All samples were analysed by: total aerobic mesophilic bacteria counts quantification in plate count agar after incubation at 30 ± 1 ºC for 72 ± 3 h; enterobacteriaceae counts quantification in violet red bile dextrose agar, being incubated at 37 ± 1 ºC for 24 h; and yeasts and moulds quantification in rose bengal chloramphenicol agar after incubation at 25 ± 1 ºC for 5 days. Petri dishes with 15-300 colony forming units (CFU) were considered for quantification and the results were expressed as logarithmic of CFU per mL of juice (log CFU/mL). The maximum load considered in this study was 6.00 log CFU/mL, while the detection limit associated with the method was 1.00 log CFU/mL. Measurements were done in triplicate for analyses of total aerobic mesophilic bacteria and enterobacteriaceae and quintuplicate for analyses of yeasts and moulds.

3.18.2. Physicochemical analyses

The pH value of the samples was measured, in triplicate, at 25 °C with a properly calibrated glass electrode (Crison, Barcelona, Spain).

The colour parameters *a** (red/green colour), *b** (yellow/blue colour), and *L** (lightness) were determined using the CIELab space, at 25 ºC. A Petri dish was filled with each carrot juice sample and placed directly under the spectrophotometer Konica Minolta CM 2300d (Minolta Konica, Japan), which was responsible to record the absorption spectra. The CIE*Lab* parameters were determined using the original SpectraMagic™ NX Software, Konica Minolta, USA, according to regulations by the International Commission on Illumination. The total colour difference variation, ΔE*, was calculated by equation 3.10:

$$\Delta E^* = [(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2]^{1/2} \quad \text{Eq. (3.10)}$$

in which ΔE* is the total colour change variation between a sample and the control (initial values identified with the subscript '0').

3.19. Statistical analysis

Each parameter was studied in triplicate being analysed three independent samples each time, except for cytotoxicity, for which five measurements were done for each sample. Statistical analysis of the results was performed using one-way Analysis of Variance (ANOVA) followed by Tukey's HSD test, at a 5% level of significance using the Minitab Statistical Software v.17.0. The results were expressed as mean ± standard deviation. The Pearson correlations were evaluated by the Pearson's correlation coefficient (R) and the statistical significance of the coefficient (p-value) using Minitab Statistical Software v.17.0.

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CHAPTER IV

RESULTS AND DISCUSSION | PROCESS OPTIMIZATION

Adapted from:

Moreira, S.A. et al (2020) - Optimization of high hydrostatic pressure assisted extraction of stinging nettle leaves using response surface methodology experimental design.

Moreira, S.A. et al (2020) - Optimization of antioxidant activity and bioactive compounds extraction of winter savory leaves by high hydrostatic pressure.

Summary

4 Overview

This chapter aims to collect all the data concerning the optimization process in order to find the best conditions for bioactive compounds extraction from stinging nettle leaves (**Section 4.1**) and from winter savory leaves (**Section 4.2**).

Both stinging nettle and winter savory are found in the Mediterranean area, being often used in traditional medicine, having several biological properties associated to its rich composition. In this work, high pressure extraction (HPE) was used to obtain extracts from both herbs. The extraction process was optimized by an experimental design via response surface methodology using a central composite face centred design. The main objective was to obtain extracts with high content of bioactive compounds and high antioxidant activity. The effects of pressure level, extraction time, and solvent concentration were evaluated, as also the impact of HPE on total phenolics (TPC), flavonoids, chlorophylls, carotenoids, and antioxidant activity.

4.1 Optimization of extraction from stinging nettle leaves

4.1.1 General aspects of models

For each dependent response (extraction yield, total phenolic compounds, total flavonoids, chlorophylls, carotenoids, and antioxidant activity by ABTS and DPPH methods) was obtained a model, which was analysed by statistical methods (see **Table 4.1**). Concerning the general goodness of fit and predictive ability of the models, three regression coefficients were studied, the regression coefficient (R^2), the adjusted regression coefficient (R^2 (aj)), and the predicted regression coefficient (R^2 (pred)). **Table 4.1** shows that these values were adequately high for all the response variables (high values are seen as an evidence for the applicability of the model in the range of variables included, being considered that a value greater than 0.75 indicates the aptness of the model **(Mohapatra et al., 2011)**). Furthermore, plots of residuals vs. the predicted response showed no defined structure and the normal probability plots of residuals exhibited a straight line as well followed a normal distribution (**Appendix D**).

4.1.2 Extraction yields

Since the extractions were performed with fresh nettle leaves, for the calculations it was considered the mass of dry matter present in nettle leaves, using the humidity (76.64%) as conversion factor between the fresh material mass and respective dry matter (**Appendix C4**). The extraction yield was greatly affected by the solvent used as can be seen in **Figures 4.1 (a-c)** and **4.2**, since as the ethanol concentration increased up to 70% (ethanol:water), the extraction yield decreased ($p<0.05$), ranging from 20.0% (for aqueous extracts) to 7.3% (for ethanolic extracts). These results indicate that the extraction yield increases with increasing polarity of the solvent used in the extraction process; this may occur due to the extraction of other compounds (such as carbohydrates and proteins, which have a higher solubility in water than in ethanol) other than phenolics and contribute to obtaining a higher yield **(Do et al., 2014; Sultana, Anwar and Ashraf, 2009)**. This is corroborated by the fact that the term 'solvent' was, by far, the most significant effect observed (presenting a F-value of 213.10 for its linear effect and a model contribution of 68% alone).

Table 4.1. Variables of response and each model characteristics for optimization of extraction from stinging nettle leaves

Variable of response and model equation	R^2 / R^2 (aj) / R^2 (pred)	Lack-of-fit (p-value and (contribution for the model construction))
Extraction yield (η)		
$\eta = 17.78 - 0.00557P + 0.592t - 0.1744S - 0.01604t^2 - 0.000463P.t + 0.000216P.S$	83.8% / 81.8% / 77.2%	0.094 (4.38%)
Total phenolic compounds (TPC)		
$TPC = 4.939 - 0.01993P + 0.3667t - 0.01624S + 0.000028P^2 - 0.01728t^2$	83.5% / 81.8% / 79.1%	0.001 (9.75%)
Total flavonoid compounds (TFC)		
$TFC = 9.75 - 0.03595P + 0.7749t - 0.1526S + 0.000049P^2 - 0.03665t^2 + 0.002329S^2$	85.6% / 84.0% / 82.0%	0.055 (3.99%)
Chlorophyll a (ChlA)		
$ChlA = 187.0 - 0.549P + 17.38t - 9.802S + 0.000734P^2 - 0.7201t^2 + 0.24653S^2 - 0.00719P.t - 0.003283P.S$	99.5% / 99.4% / 99.2%	0.001 (0.27%)
Chlorophyll b (ChlB)		
$ChlB = 779.0 - 3.439P + 43.20t - 10.464S + 0.004718P^2 - 2.089t^2 + 0.1695S^2$	92.5% / 91.6% / 90.0%	0.009 (2.68%)
Carotenoids		
$Carotenoids = 501.4 - 2.388P + 25.97t - 3.192S + 0.003251P^2 - 1.2601t^2 + 0.04679S^2 + 0.001260P.S$	93.8% / 92.9% / 91.3%	0.059 (1.56%)
Radical cation ABTS scavenging activity		
$ABTS = 13.36 - 0.04646P + 0.9473t - 0.0010S + 0.000065P^2 - 0.04451t^2 - 0.001140S^2$	95.3% / 94.7% / 93.6%	0.001 (2.98%)
Radical cation DPPH scavenging activity		
$DPPH = 12.032 + 0.00589P + 0.510t + 0.0847S - 0.02569t^2 - 0.001591S^2$	75.0% / 72.7% / 69.0%	0.002 (14.4%)

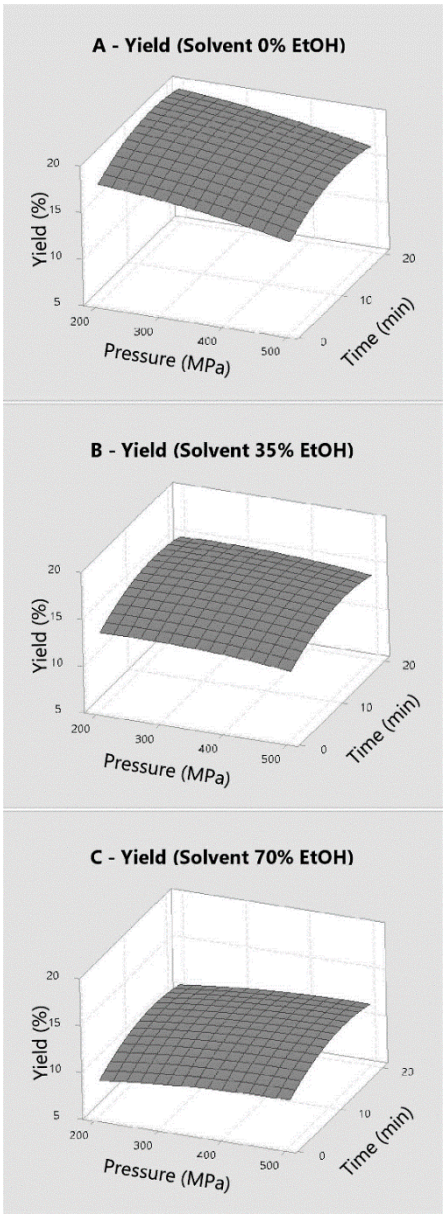

Figure 4.1. Response surface plots of extraction yield obtained for aqueous extracts (a), ethanol:water mixture at 35% (b), and ethanol:water mixture at 70% (c). The grid surfaces were predicted by the equation in **Table 4.1**

The model fitted well the experimental data since the lack-of-fitness was non-significant ($p>0.05$), and the agreement between the predicted values with the experimental values was also evaluated. The model predicted that the optimum extraction yield value would be obtained at 200 MPa, 15.6 min, using water as solvent, and the value should be 20.6%. After HPE using these conditions in a further independent assay, the extraction yield obtained was 20.1 ± 1.2%, indicating a

difference of only 2.4% ($p>0.05$) relatively to the predicted one, indicating a good prediction ability of the model concerning the experimental data.

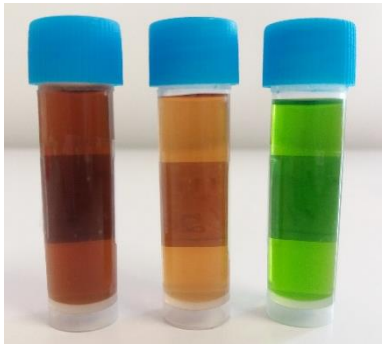

Figure 4.2. Picture of the real stinging nettle extracts obtained for each solvent mixture. From left to right: extract using 0% ethanol, extract using 35% ethanol, extract using 70% ethanol

When comparing to the extraction yield obtained at 0.1 MPa (13.3 ± 0.44%), HPE allowed to increase the value about 1.5-fold. These results indicate that HPE is clearly beneficial to increase the extraction yield of extracts from stinging nettle leaves, compared to the extraction at atmospheric pressure.

4.1.3 Total phenolic content and total flavonoids

Concerning the total phenolic compounds (TPC), 'time' was the factor with higher impact, with a F-value of 166.24. The regression coefficients for the model were all above 75% (**Table 4.1**) indicating the robustness of the model relatively to the gathered experimental data. Similar results were found to total flavonoids (TFC), since the factor with higher impact was also 'time' (quadratic term) with F-value of 219.6.

The highest TPC concentration was obtained for the aqueous extracts, at 200 MPa, and ≈10 minutes (**Figure 4.3**).

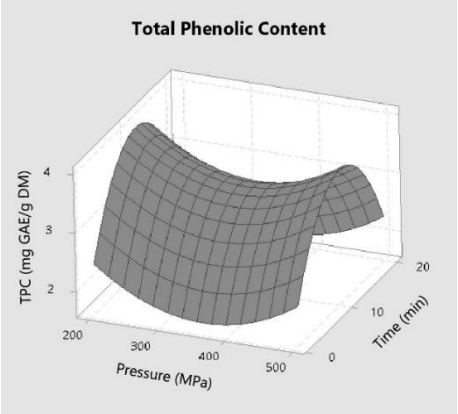

Figure 4.3. Response surface plots of total phenolic compounds obtained for aqueous extracts. The grid surfaces were predicted by the equation in **Table 4.1**

According to the obtained mathematical model, the optimal conditions for TPC extraction are 200 MPa, 10.6 min, using water as solvent, being predicted a value of 4.00 mg_{GAE}/g. Same conditions were predicted for TFC, but using 70% ethanol instead of water (**Figure 4.4**). Similar results were reported by **Vajić et al. (2015)**, who obtained a value of 7.3 mg_{GAE}/g DM in aqueous extracts from nettle leaves after maceration for 30 min. Those authors also observed that, with an increasing of ethanol concentration, they would obtain lower values of total phenolic compounds, from 7.4 to 5.1, and 0.4 mg_{GAE}/g DM for 50%, 75%, and 96% ethanol:water, respectively **(Vajić et al., 2015)**.

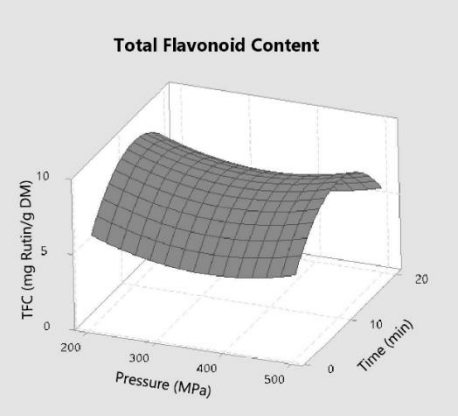

Figure 4.4. Response surface plots of total flavonoids content obtained for extracts at 70% ethanol:water. The grid surfaces were predicted by the equation in **Table 4.1**

After obtaining the optimal set of conditions and the respective predicted maximum values, those conditions were tested in a further independent assay, experimental values of 4.07 ± 0.56 mg_{GAE}/g and 9.33 ± 0.59 mg_{rutin}/g were obtained for TPC and TFC, which represents a difference of only 1.6% and 0.20 % ($p>0.05$), respectively, relatively to the predicted values. When comparing with the control extraction at 0.1 MPa, it were obtained values of only 2.2 ± 0.11 mg_{GAE}/g and 4.679 ± 0.65 mg_{rutin}/g for TPC and TFC, respectively, indicating that HPE allowed to increase phenolic compounds extraction in about 1.84-fold (an increase of 84.4%), and total flavonoids in about 2.0-fold, compared to extraction at atmospheric pressure (**Table 4.2**).

Concerning other studies on stinging nettle leaves TPC concentration, **Hudec et al. (2007)** studied fresh nettle leaves extraction and obtained values of 7.62 and 1.92 mg/g dry weight for TPC and TFC, respectively, after a maceration for 72 h, using 70% ethanol, at RT. Although HPE only extracted 70% of the TPC from nettle when

compared to **Hudec et al. (2007)**, using also 70% ethanol as solvent, the latter extraction took about 400 folds longer time. These results allow concluding that HPE is an effective and fast methodology for the extraction of phenolic compounds from stinging nettle leaves, with no need to use high temperatures, long extraction times, and organic solvents for extraction.

Table 4.2. Validation of the models and differences between the predicted and the experimental values

Variable of response	Optimal conditions (MPa/min/ % ethanol)	Predicted optimum	Experimental optimum	Control at 0.1MPa
Extraction yield (η)	200/15.6/0	20.55%	20.06 ± 1.19%	13.3 ± 0.44%
Total phenolic compounds (TPC)	200/10.6/0	4.00 mg/g	4.07 ± 0.56 mg_{GAE}/g	2.21 ± 0.11 mg_{GAE}/g
Total flavonoid compounds (TFC)	200/10.6/70	9.35 mg/g	9.33 ± 0.59 mg_{rutin}/g	4.68 ± 0.65 mg_{rutin}/g
Chlorophyll a	200/11.0/70	670.78 µg/g	752.14 ± 41.07 µg/g	313.8 ± 4.9 µg/g
Chlorophyll b	200/10.4/70	601.41 µg/g	667.66 ± 100.22 µg/g	178.0 ± 5.7 µg/g
Carotenoids	200/10.2/70	311.16 µg/g	345.48 ± 20.65 µg/g	123.2 ± 6.5 µg/g
Radical cation ABTS scavenging activity	200/10.6/0	11.70 mg/g	12.14 ± 1.67 mg_{Trolox}/g	6.84 ± 0.33 mg_{Trolox}/g
Radical cation DPPH scavenging activity	500/9.8/27	18.63 mg/g	18.08 ± 0.36 mg_{Trolox}/g	10.56 ± 0.45 mg_{Trolox}/g

4.1.4 Chlorophylls and carotenoids

Chlorophylls a (ChlA) and b (ChlB) are the two major pigments present in nettle **(Hojnik, Škerget and Knez, 2007b)**. For both responses, the term with major impact was the 'solvent', with F-values between 5058.33 and 2981.36 for ChlA and 54.13 and 215.88 for ChlB, for linear and quadratic terms, respectively. The optimal conditions predicted by the model were 200 MPa and 70% ethanol for both chlorophylls, and 11.0 min for ChlA and 10.4 min for ChlB, predicting the maximum values of 670.78 and 601.41 µg/g DM for ChlA and ChlB, respectively. The optimum obtained for those experimental conditions (in a further independent experiment) was 752.14 ± 41.07 and 667.66 ± 100.22 µg/g, indicating a difference of 12.1 and 11.0% ($p>0.05$) for ChlA and

ChlB, respectively. Comparing to the control extraction performed at 0.1 MPa, it was observed an increase of about 2.4-fold for ChlA and 3.8-fold for ChlB after HPE, since the obtained concentration at atmospheric pressure was 313.8 ± 4.9 and 178.0 ± 5.7 µg/g, respectively. These results are similar to those obtained by **Sovová et al. (2004)**, who reported values of 730 and 1000 µg/g DM for ChlA and ChlB after extraction using supercritical CO_2 as solvent, at 280 bar, 40 ºC.

Relatively to carotenoids content, its extraction behaviour followed the same pattern as chlorophylls, presenting higher extraction yields after extraction using 70% ethanol. The predicted optimum was of 311.2 µg/g after extraction at 200 MPa, for 10.2 min, 70% ethanol; and the experimental value obtained in these conditions was 345.5 ± 20.7 µg/g, representing a difference of only 11.0% ($p>0.05$), and an increase of about 2.8-fold when compared to the control extraction at 0.1 MPa (**Table 4.2**). These results present an improvement of pigments extraction from nettle leaves by HPE, when compared to extraction at atmospheric pressure. **Guil-Guerrero, Rebolloso-Fuentes and Isasa (2003)** and **Đurović et al. (2017)** obtained a value of total carotenoids of only 51.4 and 54.7 µg/g, respectively, after a extraction of fresh nettle leaves using acetone, diethyl ether, water and anhydrous sodium sulphate **(Guil-Guerrero, Rebolloso-Fuentes and Isasa, 2003)** and Soxhlet extraction with 96% ethanol **(Đurović et al., 2017)**. When comparing those results with the ones present in this article, the authors can observe that HPE allowed an increment of extraction of carotenoids from nettle leaves of more than 5-fold, without needing to use environment harmful organic solvents.

4.1.5 Antioxidant activity

The antioxidant activity of the extracts was accessed by both radical cation ABTS and DPPH scavenging activity assays, due to the simplicity of the methods, and also due to their sensitivity. ABTS assay is very time consuming, since it needs the pre-formation of the radicals by oxidation with potassium persulphate, becoming a not so reproducible method. Nonetheless, it is simple to perform, since the radical is soluble in both water and organic solvents, enabling the determination of antioxidant capacity of hydrophilic and lipophilic compounds; DPPH radical is more stable than the ABTS one, allowing better reproducibility, also being fast and simple, but it is better soluble in organic solvents, and its results only shows the antioxidant activity of lipophilic compounds. The terms with major impact for both models construction were ‘solvent’

(linear term, F-value of 445.85 for ABTS assay) and 'time' (quadratic term, F-value of 30.28 for DPPH assay). These results are according to the optimal conditions predicted by the ABTS model, since the best solvent was water (linear behaviour for this factor), the best extraction time was 10.6 min (a maximum for the intermediate value, indicating a quadratic behaviour for this dependent variable), at a pressure of 200 MPa. Concerning the maximization of the antioxidant activity by ABTS assay, the model predicted a maximum value of 11.70 mg_{Trolox}/g when using the extraction conditions 200 MPa, 10.6 min, and 0% ethanol. The experimental value obtained for those conditions (in a further independent experiment) was 12.14 ± 1.67 mg_{Trolox}/g, indicating a good fitness of the model, since the difference between the two values was only 3.8% ($p>0.05$). Also, when compared to the control extraction at atmospheric pressure, HPE allowed an increase of about 1.8-fold (representing an increase of 77.7%). Concerning the validation of the DPPH model, it predicted an optimum of 18.63 mg_{Trolox}/g for 500 MPa, 9.8 min, and 27% of ethanol. When comparing to the experimental value obtained after extraction in these conditions (in a further independent experiment), the value was 18.08 ± 0.36 mg_{Trolox}/g, representing a difference of only 3.0% ($p>0.05$), indicating that the model can predict unknown values. **Belscak-Cvitanovic et al. (2015)** also evaluated the antioxidant activity of stinging nettle extracts by ABTS assay, and observed that, after extraction by maceration for 24 h at RT, the extracts presented an antioxidant activity of only 0.28 mM_{Trolox} (equivalent of about 70.08 $\mu g_{Trolox}/mL$). In the present work, HPE allowed obtaining a much higher antioxidant activities in a shorter extraction time.

4.1.6 Correlation between dependent variables

The bioactive compounds concentration and the antioxidant activity of each extract can be correlated, and in **Table 4.3** it is possible to observe the correlation coefficients (R^2) for the linear correlation between the antioxidant activity by the ABTS and DPPH methods and the concentration of total phenolic compounds and total flavonoids for each extraction condition. The highest correlation values (**Table 4.3**) were obtained between total phenolic compounds and flavonoids for all solvents when the extraction occurred at atmospheric pressure, at 200 MPa, and at 500 MPa.

Table 4.3. Correlation values (R^2, expressed in percentage) between response variables

		Phenolics				Flavonoids			
		0.1 MPa	**200 MPa**	**350 MPa**	**500 MPa**	**0.1 MPa**	**200 MPa**	**350 MPa**	**500 MPa**
Flavonoids	**0%**	**99.9**	**89.6**	**94.0**	**99.7**	-	-	-	-
	35%	**98.5**	**99.9**	**94.3**	**99.7**	-	-	-	-
	70%	**95.3**	**99.2**	3.2	**88.5**	-	-	-	-
ABTS	**0%**	**99.8**	5.7	**94.4**	**82.5**	**99.8**	0.760	**99.9**	**78.5**
	35%	**99.6**	**99.7**	**92.9**	**99.9**	**99.7**	**99.6**	**99.9**	**99.9**
	70%	**84.5**	**99.9**	64.7	**99.8**	65.8	**99.6**	53.2	**91.3**
DPPH	**0%**	1.14	**91.7**	**93.2**	**98.5**	1.34	66.2	**99.9**	**97.0**
	35%	1.16	**89.7**	51.9	**99.7**	5.16	**89.3**	28.6	**99.9**
	70%	**96.0**	**99.0**	48.6	49.7	**83.3**	**99.9**	69.0	17.9

Note: The highest correlation values are identified in bold (above 75%).

When studying the relation between phenolic compounds and the antioxidant activity by ABTS assay, it was observed that for the aqueous extracts, the highest correlation occurred for extraction at 0.1 and 350 MPa; on the other hand, for ethanolic (70% ethanol) extracts, the highest correlation occurred for the extraction at 200 MPa. This indicates that different extraction conditions allow to obtain different compounds in the final extract. The compounds extracted at 200 MPa, that grant the antioxidant activity of the aqueous extracts are not phenolic compounds, being necessary to develop more research in this area in order to explore this possibility.

It is noteworthy that when using an intermediate solvent concentration (35% ethanol) all the correlations were extremely high. These data are corroborated by the Pearson coefficients (**Table 4.4**), being obtained a value of 0.869 ($p<0.05$) for this correlation (between ABTS assay and phenolics content). Concerning the DPPH assay, the highest correlations occurred at 200 and 500 MPa, for the extracts obtained with 0% and 70% ethanol, and although the Pearson coefficient is low (0.525), it is still significant ($p<0.05$).

Table 4.4. Pearson correlation values between response variables expressed in percentage; between parenthesis appears the p-value for each correlation

	Flavonoids	**ABTS**	**DPPH**
Phenolics	37.1 (0.006)	86.9 (0.000)	52.5 (0.000)
Flavonoids	-	16.1 (0.245)	-16.1 (0.227)
ChlA	-	-66.7 (0.000)	-62.6 (0.000)
ChlB	-	-20.4 (0.160)	-44.7 (0.001)
ChlT	-	-58.0 (0.000)	-61.6 (0.000)
Carotenoids	-	13.9 (0.340)	-17.3 (0.215)
ABTS	-	-	68.0 (0.000)

4.2 Optimization of extraction from winter savory leaves

4.2.1 General aspects of models

Different HPE conditions (pressure level, extraction time, and ethanol concentration) were applied in order to experimentally obtain several responses (total phenolics, total flavonoids, chlorophylls, carotenoids, and extraction yield; as well as for three antioxidant activity assays: radical scavenging by ABTS and DPPH assays, and ferric reducing antioxidant power). It was possible to fit all experimental values into second-order polynomial models provided by a central composite experimental design (see **Table 4.5**), being characterized by non-significant ($p>0.05$) lack-of-fit values and low residuals percentage contribution for model construction, indicating that the models are well fitted to the experimental data. Residual plots, also known as diagnostic plots, for each response were also performed (normal probability plot, histogram, residuals versus fit, and residuals versus order) (see example for total phenolic compounds in **Figure 4.5**). These plots indicate the goodness-of-fit of the models in regression and ANOVA and should help to determine if the ordinary least squares assumptions are being met. If these assumptions are satisfied, then ordinary least squares regression will produce an unbiased coefficient estimative with the minimum variance.

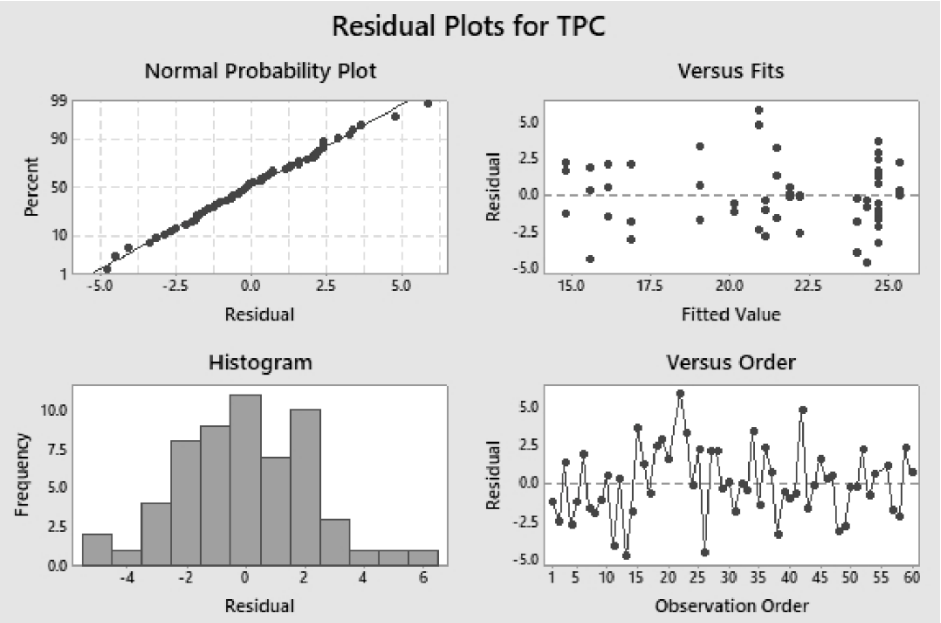

Figure 4.5. Example of residual plots (designed by Minitab Statistical Software, version 17.0) for total phenolic compounds

Table 4.5. Variables of response and model characteristics for optimization of extraction from winter savory leaves

	F-value		p-value		R^2 (%)
Variable of response and model equation	**Model**	**Lack-of-fit**	**Model**	**Lack-of-fit**	
Extraction yield (η)					
$\eta = 20.0 - 2.17x10^{-3}P - 3.71x10^{-1}t - 1.65S + 1.49x10^{-2}t^2 + 3.08x10^{-4}PS$	30.10	0.29	0.000	0.954	86.98
Total phenolic compounds (TPC)					
$TPC = 5.42 + 1.01x10^{-1}P + 6.99x10^{-2}t + 9.57x10^{-2}S - 1.41x10^{-4}P^2 - 2.46x10^{-3}S^2$	24.51	1.70	0.000	0.117	80.21
Total flavonoid compounds (TFC)					
$TFC = 2.02 + 8.56x10^{-2}P - 5.04x10^{-1}t - 2.88x10^{-2}S - 1.21x10^{-4}P^2 + 2.23x10^{-2}t^2$	6.92	1.54	0.000	0.165	80.43
Chlorophyll a (ChlA)					
$ChlA = 95.4 - 3.89x10^{-1}P + 2.10t - 3.93S + 6.18x10^{-4}P^2 - 7.20x10^{-2}t^2 + 1.00x10^{-1}S^2 - 3.05x10^{-2}tS$	553.34	14.48	0.000	0.010	98.85
Chlorophyll b (ChlB)					
$ChlB = 102.6 + 2.54x10^{-2}P - 1.45t - 4.84S + 1.42x10^{-1}t^2 + 7.56x10^{-2}S^2 - 3.92x10^{-2}tS$	164.89	1.12	0.000	0.371	95.46
Carotenoids					
$Carotenoids = 115.2 - 1.67x10^{-1}P + 3.11x10^{-1}t - 2.52S + 1.59x10^{-4}P^2 + 2.34x10^{-2}S^2 + 2.58x10^{-3}PS$	66.05	2.23	0.000	0.057	89.60
Radical cation ABTS scavenging activity					
$ABTS = 35.5 + 1.84x10^{-1}P - 2.41t + 1.21S - 2.73x10^{-4}P^2 + 1.26t^2 - 2.15x10^{-2}S^2 + 4.10x10^{-4}PS$	35.87	2.37	0.000	0.059	83.67
Radical cation DPPH scavenging activity					
$DPPH = 75.1 - 9.19x10^{-2}P - 1.35t - 3.29x10^{-1}S + 3.02x10^{-3}S^2 + 6.14x10^{-3}Pt$	25.99	1.86	0.000	0.084	81.42
Ferric reducing antioxidant power (FRAP)					
$FRAP = 218.9 + 6.26x10^{-1}P + 8.32x10^{-1}t + 1.69S - 1.06x10^{-3}P^2 - 3.21x10^{-2}S^2 + 1.89x10^{-3}PS$	19.26	2.90	0.000	0.062	81.09

The normal probability plot and the histogram help to visualize if there are outliers in the experimental data, while the residuals versus fit and residuals versus order indicate if the residuals have a constant variance and are not correlated to each other, meaning that the error assumed for the model construction is minor and does not interfere with the prediction of other values and conditions. Considering all the obtained statistical parameters, the experimental data were used to create response surface graphs and mathematical models. Relatively to each model p-value, all of them are significant ($p<0.05$), which means that at least one of the terms of each model has an impact on the mean response.

After each model construction, an optimization process was conducted in order to assess the optimal conditions for extraction and the predicted maximum value for each response variable (**Table 4.6**). It is noteworthy that for each response variable it was obtained a different set of optimum extraction conditions. As an attempt to define extraction conditions that would allow obtaining extracts with overall optimum bioactivities, in what concerns all the response variables studied (differing from the individual optimum by less than 10%), for each ethanol concentration (0, 35, and 70% ethanol), a comparison exercise using Minitab Statistical Software response optimizer function was performed. It was calculated the maximum value for each response variable, using the optimal conditions obtained for each of the other response variables (see **Table 4.7**). After, the predicted values so obtained were compared (by percentage) with their own predicted individual optimum values and the different response variables were clustered (considering a difference <10%) for each ethanol concentration studied (0, 35, and 70% ethanol). This way, three sets of general experimental conditions (taking into account if the values would differ less than 10% from the optimal individual value) were obtained: 500 MPa, 20 min, 0% ethanol for extraction yield and antioxidant activity by DPPH; 348 MPa, 20 min, 35% ethanol for total phenolic and flavonoid compounds, and antioxidant activities by ABTS and FRAP; and 500 MPa, 1 min, 70% ethanol for pigments (chlorophylls a and b and carotenoids).

Finally, an experimental validation process was conducted, which consisted on an independent extraction experiment, using the predicted optimal conditions that led to an experimental confirmation of the maximum values with a deviation of ±10% (**Table 4.6**).

Table 4.6. Validation of the models and differences between the predicted and the experimental values

Variable of response	Optimal conditions (MPa/min/% ethanol)	Predicted optimum	Experimental optimum	RSD* (predicted and experimental)	Control at 0.1MPa	Increase (experimental *vs* control)
Extraction yield	350/1/0	18.93%	18.18 ± 1.83%	2.85%	9.23 ± 1.97%	96.90%
Total phenolic compounds	358/20/19.5	25.34 mg/g	23.62 ± 5.09 mg_{GAE}/g	11.5%	15.75 ± 1.36 mg_{GAE}/g	39.95%
Total flavonoid compounds	351/1/0	16.61 mg/g	14.22 ± 0.83 mg_{rutin}/g	11.01%	9.20 ± 1.84 mg_{rutin}/g	54.53%
Chlorophyll a	500/1/70	272.48 µg/g	251.75 ± 18.33 µg/g	5.59%	179.11 ± 8.32 µg/g	40.55%
Chlorophyll b	500/1/70	143.24 µg/g	148.63 ± 16.73 µg/g	2.61%	112.68 ± 4.86 µg/g	31.90%
Carotenoids	500/20/70	106.29 µg/g	103.85 ± 14.22 µg/g	1.64%	69.15 ± 2.99 µg/g	50.18%
Radical cation ABTS scavenging activity	360/20/31.8	90.12 mg/g	88.14 ± 9.35 mg_{Trolox}/g	1.56%	51.91 ± 1.62 mg_{Trolox}/g	69.80%
Radical cation DPPH scavenging activity	500/20/0	63.51 mg/g	64.82 ± 6.42 mg_{Trolox}/g	1.44%	43.66 ± 6.52 mg_{Trolox}/g	48.46%
Ferric reducing antioxidant power	327/20/36	368.17 mg/g	336.21 ± 48.19 mg_{AIS}/g	7.31%	260.58 ± 29.78 mg_{AIS}/g	29.02%

*RSD means a Relative Standard Deviation between two values (a value lower than 10% indicates no differences between the values)

Table 4.7. Comparison of optimal extraction condition set for each individual response and general final models

Variable of response	Optimal individual conditions set (MPa/min/% ethanol)	Predicted optimum	Optimal general conditions set (MPa/min/% ethanol)	Predicted optimum	Difference between predicted values (%)
Extraction yield	350/1/0	18.93%	500/20/0	17.49%	-8
Total phenolic compounds	358/20/19.5	25.94 mg/g	348/20/35	25.34 mg/g	-2
Total flavonoid compounds	351/1/0	16.61 mg/g	348/20/35	14.95 mg/g	-10
Chlorophyll a	500/1/70	272.48 µg/g	500/1/70	272.48 µg/g	0
Chlorophyll b	500/1/70	143.24 µg/g	500/1/70	143.24 µg/g	0
Carotenoids	500/20/70	106.29 µg/g	500/1/70	100.38 µg/g	-6
Radical cation ABTS scavenging activity	360/20/31.8	90.12 mg/g	348/20/35	89.79 mg/g	0
Radical cation DPPH scavenging activity	500/20/0	63.51 mg/g	500/20/0	63.51 mg/g	0
Ferric reducing antioxidant power	327/20/36	368.17 mg/g	348/20/35	367.60 mg/g	0

4.2.1 Extraction yields

HPE implies the use of high hydrostatic pressure, which leads to a higher differential pressure between the inner and exterior of the cell, allowing the solvent permeation to occur faster than in conventional extraction; this particular characteristic allied to the easier breakage of the cell wall and consequent higher volume of solvent inside the cell, HPE is expected to increase extraction yield, while it decreases extraction time and the energy needed for the process to occur **(He et al., 2011; Lee, He and Ahn, 2010; Qadir et al., 2009; Xi et al., 2013)**.

The choice of solvent is considered an upmost parameter for any extraction process, being dependent of the solubility of the compounds of interest, as well as, its interaction with the sample matrix. Ethanol is one of the most used solvents for extraction of phenolic compounds, especially due to its moderate polarity (ε = 25.5 at 20°C) and easy removal from the final extract **(Shouqin, Xi and Changzheng, 2005)**. In **Figure 4.6 (a)** it is possible to observe that extraction yield does not seem to be affected by extraction time, meaning that lower extraction times (1 min) allow to obtain similar yields than higher extraction times (20 min); nevertheless, this parameter is highly affected by ethanol concentration, indicating that for aqueous extracts (0% ethanol), independently of pressure level, the expected extraction yield is about 20%, while for ethanolic extracts (70% ethanol), to obtain a yield of ~20% it is necessary to use a pressure level of about 500 MPa.

According to the mathematical model, the optimal conditions to obtain a maximum predicted yield value of 18.93% are 350 MPa, 1 min, 0% ethanol. When applying these conditions in an independent experiment, it was possible to obtain a value of 18.18 ± 1.83%, representing a deviation of the predicted value of only 2.85%. These results indicate that the model was well-fitted to the experimental data, being able to correctly predict conditions and probable values for extraction yield.

4.2.2 Extracts characterization

After extraction with the different ethanol mixtures, time of extraction and pressure levels, the extracts were studied relatively to their content in total phenolic compounds (TPC), total flavonoids content (TFC) and pigments (individual chlorophylls (ChlA and ChlB) and carotenoids). In **Figure 4.6** it is possible to see some examples of the surface plots obtained in the present study. **Figure 4.6 (b)** and **Figure 4.6 (c)** indicate that total

phenolics and total flavonoids are better extracted with the solvent 35% ethanol (mixture of ethanol:water, v/v), and that are not so affected by time of extraction or pressure level (although the plots present a slight curve, indicating that mild pressures could improve those compounds extraction.

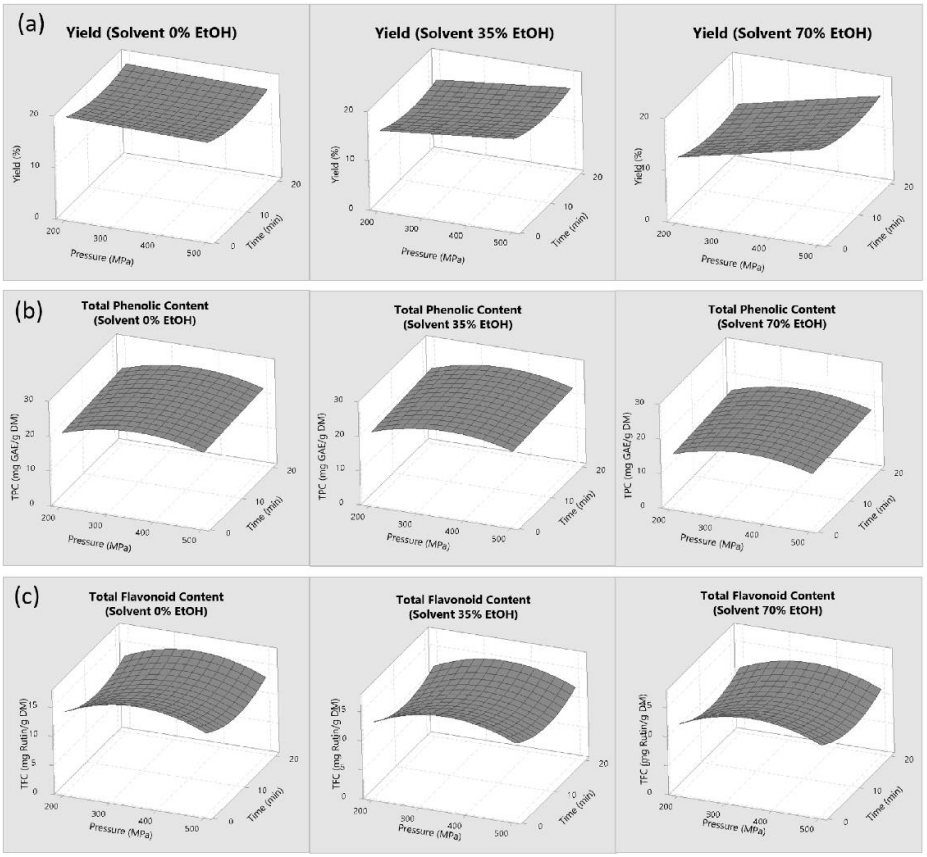

Figure 4.6. Response surface plots obtained by experimental design using a central compositive face-centred design of (a) extraction yield, (b) total phenolic compounds, and (c) total flavonoids. The grid surfaces were predicted by the equations showed in **Table 4.5**

These data are corroborated by the results in **Table 4.7**, where it is possible to see that the optimal conditions predicted by the TPC model were 358 MPa, 20 min, 19.5% ethanol, while for extraction of total flavonoids, the predicted conditions set was 351 MPa, 1 min, 0% ethanol (aqueous extracts). As stated above, in **section 4.2.1 'General aspects of models'**, in order to obtain extracts with overall optimum bioactivities (differing from the individual optimum by less than 10%), it was calculated which set of conditions would allow to obtain extracts with higher concentration of the compounds quantified. Thus, total phenolic compounds and flavonoids optimized extract was obtained at 348 MPa, 20 min, 35% ethanol, with predicted values of 25.94 mg_{GAE}/g and 16.61 mg_{rutin}/g, respectively.

Furthermore, after performing an independent experiment using these conditions, it were obtained the experimental values of 23.62 ± 5.09 mg_{GAE}/g for total phenolics and 14.22 ± 0.83 mg_{rutin}/g for flavonoids, indicating that the model was validated, as well were the "optimal general condition sets".

These results are according to the ones reported by **López-Cobo et al. (2015)** who obtained a value of 25.82 ± 3.14 mg_{GAE}/g for total phenolics after a 2-step-solid-liquid extraction using three organic solvents (methanol, ethanol, and acetone) in an ultrasonic bath for 10 minutes. Also **Zekovic et al. (2017b)** tried to optimize the extraction process using microwaves, being able to obtain a maximum recovery of total phenolics of only 0.0744 mg_{GAE}/g DW and total flavonoids of only 0.0481 $mg_{catechin}/g$ DW after extraction using 70% ethanol for 12.5 min. Furthermore, when comparing the results in the present study with the ones reported by **Ćetković et al. (2007b)** who obtained a value of only 1.358 ± 0.0679 mg_{GAE}/g for total phenolics using a 6-step-liquid-solid extraction with n-butanol as solvent, for 48 h, it is clear that HPE can be seen as a fast and environmentally-friendly technology, which allows to obtain similar to higher extraction values, using non-pollutant solvents.

The same line of reasoning was applied in order to optimize de extraction of pigments (chlorophylls and carotenoids) from winter savory leaves (**Figure 4.7**). After obtaining a general set of conditions (500 MPa, 1 min, 70% ethanol), the differences between the predicted and the experimental optimum values were below 6%, indicating that also the three models obtained for the extraction of pigments were validated and, for so, well fitted to the experimental data.

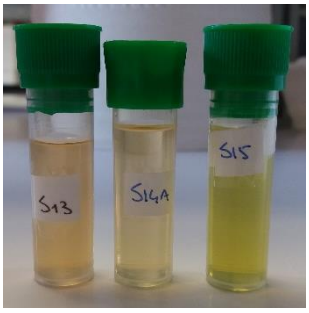

Figure 4.7. Picture of the real winter savory extracts obtained for each solvent mixture. From left to right: extract using 0% ethanol, extract using 35% ethanol, extract using 70% ethanol

4.2.3 Antioxidant activity

There are several available assays to estimate the antioxidant activity of herbal extracts, such as ABTS and DPPH radical scavenging assays, and ferric reducing antioxidant power, but there are few studies which help to compare and correlate the different methods **(Thaipong et al., 2006)**. These three methods were of choice due to their simplicity, sensitivity, and reproducibility. Each method has its own characteristics: (1) ABTS assay is very time consuming, since it needs the pre-formation of the radicals by oxidation with potassium persulphate, thus not being reproducible. Nonetheless, it is a simple method, since the radical is soluble in both water and organic solvents, enabling the determination of antioxidant capacity of hydrophilic and lipophilic compounds, being widely reported in the literature; (2) DPPH radical is more stable than the ABTS one, allowing better reproducibility, also being fast and simple, but it is better soluble in organic solvents, and its results only shows the antioxidant activity of lipophilic compounds; (3) FRAP, although simple and reproducible, is a more time consuming method. Nevertheless, FRAP assay essentially measures the reducing potential of an antioxidant reacting with a ferric complex, following the principles of ABTS, but occurs at an acidic and not neutral pH **(Shah and Modi, 2015)**.

While the solvent parameter was the one with the major impact for ABTS and FRAP assays, for DPPH method it was the interaction between pressure and time the one with major impact on the construction of the models (as can be seen in **Figure 4.8 (a-c)**). These results are corroborated by the fact that the optimal sets of conditions predicted for maximizing ABTS and FRAP assays were both 348 MPa, 20 min, 35% ethanol, with a predicted value of 89.79 and 367.60 mg/g DW, respectively, while for DPPH the optimal set of conditions was 500 MPa, 20 min, 0% ethanol, with a prediction of 63.51 mg/g DW. All the models were validated, since after performing the independent experiments with the predicted conditions, it were obtained experimental values with differences not higher than 7.5% relatively to the predicted values. The results reported in this work contrast to the ones of **Hajdari et al. (2016)** who studied the antioxidant activity of *S. montana* leaves extracts using DPPH and FRAP assays, and obtained maximum values of 342.9 and 11.4 mg_{Trolox}/g DW, respectively. This difference can be attributed to the extraction method used, since **Hajdari et al. (2016)** used 50% methanol, which enabled the extraction of a

higher concentration of lipophilic compounds, and consequently, a higher value for DPPH assay.

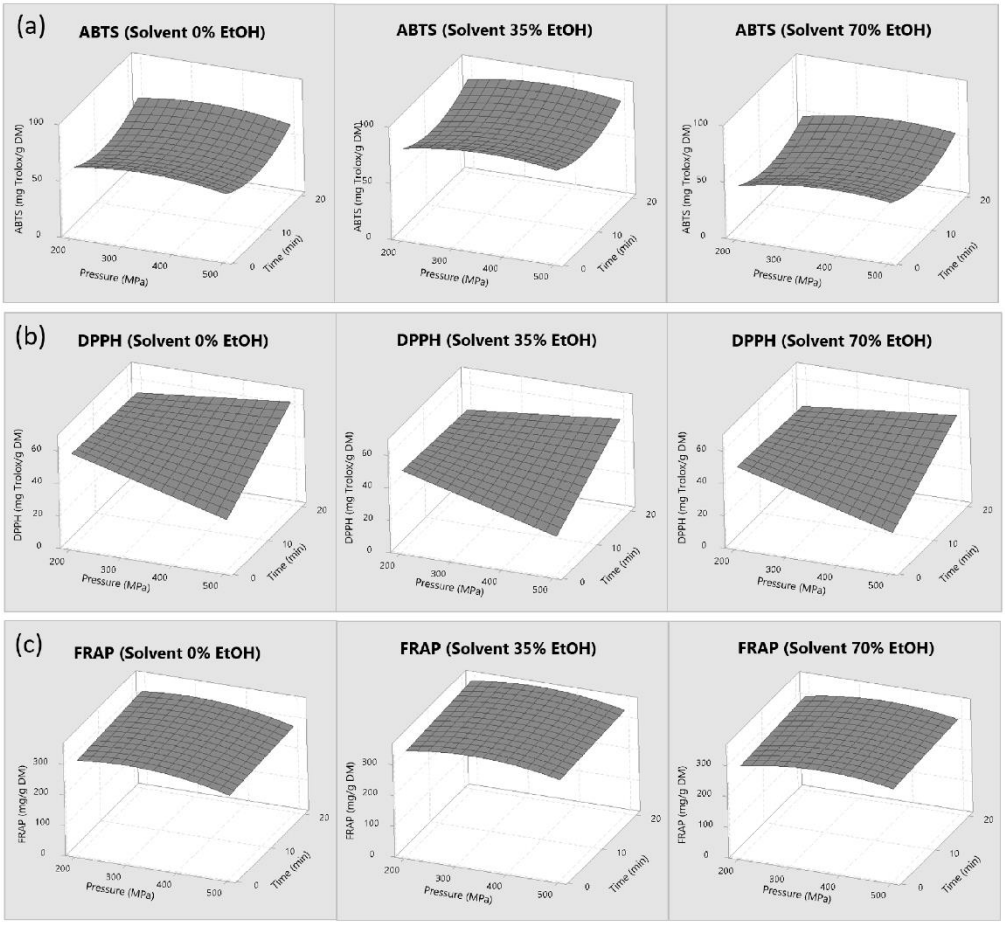

Figure 4.8. Response surface plots obtained by experimental design using a central compositive face-centred design of antioxidant activities by (a) ABTS assay, (b) DPPH assay, and (c) FRAP assay. The grid surfaces were predicted by the equations showed in **Table 4.5**

Contrasting results were also found against the ones reported by **Gião et al. (2009)**, who stated that longer extraction times led to higher antioxidant activity measured by ABTS assay. In the present work, it was possible to observe that, for ABTS and FRAP assays, time was the parameter that had the minor impact in the final antioxidant capacity (see **Figure 4.8 (a-c)**).

4.2.4 Correlation between dependent variables and comparison to control extraction

The two correlation coefficients tested in the present work allowed to measure the extent (strength and direction) at which two responses tend to change together. While the Pearson correlation estimates if two variables have a linear relationship, *i.e.* both variables change at a constant rate, the Spearman correlation analyses the possibility of the variables to have any relationship, *i.e.*, if they change together, but not at a constant rate **(Hauke and Tomasz, 2011; Minitab, 2019)**. In **Table 4.8** it is possible to see that the antioxidant activity measured by ABTS and FRAP assays seems to be the one which correlate (for both Pearson and Spearman correlations) better with total phenolic compounds, although the higher correlation values were obtained for pigment content, in special for total chlorophylls. This fact can be attributed to the not-so-studied fact that chlorophylls can be involved in the antioxidant mechanisms of plants. Nevertheless, that mechanism of action it is still not very clear and may be not related to the capacity of hydrogen donation but to the prevention of the decomposition of hydroperoxides **(Lanfer-Marquez, Barros and Sinnecker, 2005)**. It was observed a clear lack of correlation between the known antioxidant compounds (phenolics and flavonoids) and the antioxidant activity measured by all the assays. Similar results were reported by **Vladić et al. (2017)** who stated that the possible generation/extraction of new bioactive compounds with antioxidant properties via Maillard, caramelization, and thermo-oxidation reactions could lead to higher antioxidant activity of the final extract, with no visible correlation to the extracted compounds. Nevertheless, since in this work no high temperatures were used, it can be assumed that high pressure helped to extract new/other compounds that helped to improve extract's antioxidant capacity.

Concerning the comparison of HPE process with the control extracts at 0.1 MPa, a maximum extraction yield value of 9.23 ± 1.97% was obtained for the control, while it was possible to observe an increase ($p>0.05$) of about 96.9% after using HPE. It is also noteworthy that when comparing HPE to the control extraction, it was observed an increase of 40% for total phenolic compounds, and of 55% for flavonoids for the HPE extracts, as well as an increase ($p>0.05$) for all three antioxidant activity assay (29, 48, and 70% increase for FRAP, DPPH, and ABTS, respectively), indicating that this methodology can efficiently improve extracts quality.

Table 4.8. Pearson and Spearman correlation values between response variables; between parenthesis appears the p-value for each correlation. It should be noted that the table is labelled with a colour code (only the significant correlations ($p<0.05$) are highlighted in grey cells, and more intense colour (three levels) represents a higher correlation value

	Pearson correlations					**Spearman correlations**				
	Flavonoids	**ABTS**	**DPPH**	**FRAP**	**Yield**	**Flavonoids**	**ABTS**	**DPPH**	**FRAP**	**Yield**
Phenolics	0.473 (0.000)	0.663 (0.000)	0.086 (0.531)	0.499 (0.000)	0.358 (0.012)	0.433 (0.001)	0.657 (0.000)	-0.007 (0.959)	0.481 (0.000)	0.241 (0.095)
Flavonoids	-	0.419 (0.002)	0.305 (0.023)	0.145 (0.305)	0.483 (0.000)	-	0.381 (0.004)	0.361 (0.007)	0.103 (0.466)	0.483 (0.000)
ChlA	-	-0.710 (0.000)	0.017 (0.907)	-0.437 (0.002)	-0.249 (0.091)	-	-0.708 (0.000)	0.104 (0.466)	-0.565 (0.000)	0.245 (0.097)
ChlB	-	-0.717 (0.000)	0.184 (0.193)	-0.774 (0.000)	0.120 (0.423)	-	-0.670 (0.000)	0.204 (0.146)	-0.667 (0.000)	0.187 (0.208)
ChlT	-	-0.788 (0.000)	0.048 (0.741)	-0.619 (0.000)	-0.190 (0.207)	-	-0.757 (0.000)	0.133 (0.353)	-0.597 (0.000)	0.068 (0.654)
Carotenoids	-	-0.631 (0.000)	0.217 (0.127)	-0.572 (0.000)	0.376 (0.009)	-	-0.710 (0.000)	0.204 (0.152)	-0.582 (0.000)	0.358 (0.014)
ABTS	-	-	0.087 (0.530)	0.550 (0.000)	-	-	-	0.065 (0.636)	0.617 (0.000)	-
DPPH	-	-	-	-0.113 (0.423)	-	-	-	-	-0.206 (0.143)	-

4.3 Conclusions

The main results of these studies indicate that high pressure assisted extraction can be effectively used to increase the concentration of total phenolic compounds, total flavonoids, individual and total chlorophylls, and carotenoids, while it was also observed a clear improvement of antioxidant activity by ABTS, DPPH, and FRAP assays. In addition, the use of a response surface methodology coupled to mathematical models, helped predicting the optimal extraction conditions and calculate the maximum values for each response variable. This enabled the possibility to construct optimal set of conditions for each solvent concentration, which facilitate the possibility of obtaining different extracts, which one with desirable characteristics. Ethanol concentration was the variable with the highest impact in all extractions, followed by pressure level, and extraction time. This indicates that HPE can operate in a considerably short extraction time, since this parameter was the one with lower F-value, thus had fewer impact on the process; for so, a quicker extraction production with improved properties is an indicator that this innovative and environmentally-friendly (produces high quality extracts with no organic solvents in the end, since ethanol is easily recycled) extraction method is a promising alternative for the conventional extraction techniques.

The gathered data highlighted the superiority of the HPE extraction over the control extractions, and the optimized conditions obtained in these works are important to promote unknown herbs valorization since high-pressure increased antioxidant activity and the content of all classes of total compounds quantified. However, in order to understand the impact of the increase of bioactive compounds content is necessary to study the effect of HPE on different biological activities, such as antioxidant in the presence of a biologically relevant radical source (ORAC assay), the ability to protect DNA in the presence of a damaging system, and also to access the extracts cytotoxicity.

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CHAPTER V

RESULTS AND DISCUSSION | BIOLOGICAL ACTIVITIES

Adapted from:

Moreira, S.A. et al (2020) - Effect of high hydrostatic pressure extraction on biological activities from stinging nettle extracts. Food and Function. doi: 10.1039/c9fo02442e

Moreira, S.A. et al (2020) - Effect of high hydrostatic pressure extraction on biological activities and phenolics composition of winter savory leaves extracts.

Summary

5 Overview

This chapter aims to collect all the data concerning the analysis of biological activities from the optimized extracts from stinging nettle (**Section 5.1**) and from winter savory leaves (**Section 5.2**) obtained in **Chapter IV.**

Stinging nettle is traditionally used by several as a therapeutic herb and winter savory is known to have several biological properties such as antimicrobial, antioxidative, and antiproliferative, related to its rich composition of secondary metabolites. In the present Chapter, the biological properties and cytotoxicity of the optimized extracts obtained by high pressure assisted extraction (HPE) were studied and compared with similar extracts obtained with same solvent under normal pressure conditions. Each extract was characterized for their individual compounds profile and different biological properties, such as antioxidant activity, pro-oxidant activity (DNA degradation capacity), antihypertensive activity, antimicrobial and antibiofilm properties, as also as cytotoxicity and anti-proliferative effect on human tumour cell lines.

5.1 Biological activities of extracts obtained from stinging nettle leaves

5.1.1 Individual compounds

Phenolic composition of herbs can be affected by several factors, such as variety and genotype of the plant, climate, harvest time, storage, processing or even the treatment of the plant **(Dobrinas, Stanciu and Lupsor, 2017)**. The total phenolic content of the extracts under study was already described and discussed on **Chapter IV, Section 4.1**. Generally, the control extracts (extraction at 0.1 MPa, using 0-70% ethanol as solvent) had lower phenolics concentration than the extracts obtained after HPE (Table 3), showing the highest total phenolic content registered for the aqueous extracts after HPE at 200 MPa (585.26 ± 26.57 mg GAE/100 g DM) (**Chapter IV, Section 4.1)**. In the eight extracts under study (see **Table 3.1**, **Chapter III**, for nomenclature) it was possible to identify and quantify 12 phenolic compounds and among the phenolic acids, isoferulic acid was the one with higher concentration ($p<0.05$), with values ranging from 1.59 ± 0.19 to 116.56 ± 11.68 mg/100 g, while *p*-coumaroylmalic acid isomers and chlorogenic acid were found at lower levels ($p<0.05$). Concerning the flavonoids, rutin was the only one, which was possible to detect and quantify (**Table B2, Appendix B** (for calibration curves) and **Table 5.1**), mainly in ethanolic extracts (N200/10.6/35, N0.1/10.6/35, N200/10.6/70, and N0.1/10.6/70). Several studies indicate that a high content of hydroxycinnamic acids, such as chlorogenic and caffeic acids, as well as flavonoids, is an important feature of an extract since these compounds are highly correlated with antioxidant, anti-inflammatory, antimicrobial, and anticancer activities **(Zenão et al., 2017)**.

N200/10.6/0 (extract obtained at 200 MPa, 0% ethanol) was the only one where *p*-coumaric acid could be found, in a concentration of 10.27 ± 0.23 mg/100 g DM. Also **Pinelli et al. (2008)** were able to identify *p*-coumaric acid in fresh nettle samples (after an extraction at room temperature, using 70% ethanol:water (v/v) as solvent) in a concentration of 5.20 mg/100 g). This indicates that HPE can effectively perform the extraction of some phenolic compounds with no need of toxic organic solvents for extraction. Furthermore, the extracts obtained after HPE at 200 MPa, using 35 and 70% of ethanol, were the ones with more individual compounds identified. This is probably due to the non-polar nature of phenolic compounds **(Lin et al., 2016)**.

Table 5.1. Quantitative profile of phenolic and flavonoid compounds found in stinging nettle extracts. Results are shown as mg/100 g DM. Different letters indicate significant differences ($p<0.05$) between extracts for each condition set (differences analysed by row)

Identification of extracts	200 MPa 10.6 min 0% Ethanol	0.1 MPa 10.6 min 0% Ethanol	500 MPa 10.0 min 25% Ethanol	0.1 MPa 10.0 min 25% Ethanol	200 MPa 10.6 min 35% Ethanol	0.1 MPa 10.6 min 35% Ethanol	200 MPa 10.6 min 70% Ethanol	0.1 MPa 10.6 min 70% Ethanol
Total Phenolic compounds	585.3 ± 26.57	255.8 ± 30.87	285.7 ± 23.81	81.44 ± 10.10	174.2 ± 6.795	80.70 ± 8.068	398.1 ± 71.38	111.8 ± 4.301
Neochlorogenic acid (3-caffeoylquinic acid)	n.d.	n.d.	n.d.	n.d.	0.28 ± 0.06 c	n.d.	23.77 ± 3.04 a	9.39 ± 1.19 b
Caftaric acid	n.d.	n.d.	1.77 ± 0.22 c	n.d.	1.38 ± 0.03 c	n.d.	6.55 ± 0.38 a	3.38 ± 0.30 b
5-*p*-Coumaroylquinic acid	3.05 ± 0.32 d	2.68 ± 0.01 d	12.60 ± 1.08 b	n.d.	18.27 ± 1.75 a	13.10 ± 0.17 b	21.02 ± 1.11 a	7.63 ± 0.90 c
Chlorogenic acid (5-caffeoylquinic acid)	n.d.	n.d.	0.40 ± 0.08 c	n.d.	1.56 ± 0.44 c	n.d.	108.04 ± 7.30 a	40.99 ± 4.32 b
Fertaric acid	2.83 ± 0.07 b	1.85 ± 0.16 b	6.92 ± 1.10 a	n.d.	7.47 ± 1.45 a	3.14 ± 0.04 b	9.90 ± 1.36 a	2.87 ± 0.08 b
4-*p*-Coumaroylquinic acid	n.d.	n.d.	10.82 ± 0.80 b	n.d.	10.28 ± 1.41 b	8.09 ± 0.38 bc	14.87 ± 1.72 a	6.26 ± 0.27 c
2-*O*-Caffeoylmalic acid	n.d.	n.d.	5.71 ± 0.12 b	n.d.	7.84 ± 0.30 b	n.d.	49.86 ± 2.91 a	9.72 ± 0.15 b
***p*-Coumaric acid**	10.27 ± 0.23 a	6.36 ± 0.46 b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Rutin	n.d.	n.d.	0.71 ± 0.03 c	n.d.	1.28 ± 0.03 c	0.22 ± 0.03 c	64.88 ± 4.45 a	30.19 ± 0.40 b
***p*-Coumaroylmalic acid Isomer 1**	12.20 ± 0.85 bc	10.21 ± 0.20 c	15.28 ± 1.47 b	n.d.	29.30 ± 1.12 a	15.84 ± 1.08 b	15.98 ± 1.43 b	6.24 ± 0.26 d
***p*-Coumaroylmalic acid Isomer 2**	4.76 ± 0.13 bc	4.31 ± 0.14 bcd	2.80 ± 0.14 cd	n.d.	8.14 ± 0.62 a	6.15 ± 0.91 ab	7.85 ± 1.31 a	2.25 ± 0.12 de
Isoferulic acid	36.65 ± 3.49 c	34.56 ± 1.72 c	96.92 ± 11.72 a	1.59 ± 0.19 d	116.56 ± 11.68 a	67.83 ± 0.43 b	113.36 ± 4.06 a	59.27 ± 10.90 bc

Note: n.d. means that the compound was not detected.

Nevertheless, it is interesting to note that although the extract N200/10.6/0 was the one with higher concentration of total phenolic compounds, it was the extract N200/10.6/70 the one who presented higher quantity of individual compounds such as chlorogenic, caftaric, fertaric and rutin. The chromatograms of all samples were registered at 320 nm and indicated the presence of several individual phenolic compounds in different concentrations. Isoferulic acid was the only compound identified in all studied extracts. The highest concentration ($p<0.05$) of isoferulic acid was determined in N200/10.6/35 (116.56 ± 11.68 mg/100 g DM) and the lowest concentration in N0.1/10/25 (1.59 ± 0.19 mg/100 g DM), indicating a clear improvement of individual compounds extraction using HPE. Chlorogenic and neochlorogenic acids, known for their cardioprotective and anti-diabetic effects were only identified in extracts N200/10.6/35, N200/10.6/70, and N0.1/10.6/70 (extracts with 35 and 70% ethanol). The highest value ($p<0.05$) was registered for extracts obtained at 200 MPa, 70% ethanol (N200/10.6/70) with a value of 108.04 ± 7.30 mg/100 g DM. These results are according to the ones obtained by **Orcic et al. (2014)**, who reported a concentration of 123 mg/100 g of chlorogenic acid after an extraction with methanol for 48 hours using nettle leaves. These results allow concluding that HPE is an effective and fast methodology for the extraction of individual compounds from stinging nettle leaves. Relatively to rutin, the flavonoid found in the highest concentration ($p<0.05$) in the extracts, showed the highest concentration once more in N200/10.6/70 (64.88 ± 4.45 mg/100 g DM). These results were a clear improvement comparatively to **Pinelli et al. (2008), Otles and Yalcin (2012),** and **Zenão et al. (2017)** who obtain, with 70% ethanol and 80% methanol as solvents, a rutin concentration of 17.30, 19.11, and 10.60 mg/100 g, respectively.

5.1.2 Antioxidant activity

Different assays are frequently used to estimate antioxidant capacities of several compounds, such as ABTS●+ (2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)) and DPPH●+ (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assays, FRAP (ferric reducing antioxidant power), and ORAC (oxygen radical antioxidant capacity) methods. Although ABTS●+ and DPPH●+ assays are easier and faster to use when screening for antioxidant properties, they lack some biological context, since they typically disregard the molecules that the antioxidants could be protecting, such as the DNA molecule; some

authors state that ORAC assay is considered to be more pertinent and that better resembles the biological systems **(Prior et al., 2003; Thaipong et al., 2006)**. This method measures the ability of antioxidant compounds present in the extracts under study to protect a fluorescent molecule from damage by free radicals. For so, ORAC assay is known from mimicking the antioxidant activity of phenolic compounds in biological systems, since it uses biologically relevant free radicals and integrates both time and the degree of antioxidants activity **(Lucas-Abellán et al., 2011)**.

For so, the antioxidant capacity of the studied extracts was quantified by ORAC assay. In **Table 5.2** it is possible to observe that among aqueous extracts (N200/10.6/0 (HPE) and N0.1/10.6/0 (control)) and 25% ethanol:water extracts (N500/10/25 (HPE) or N0.1/10/25 (control)) there were no significant differences ($p>0.05$). Nevertheless, when analysing the extracts obtained using 35 and 70% ethanol (N200/10.6/35 and N0.1/10.6/70), it is possible to observe a clear improvement ($p<0.05$) of antioxidant capacity after stinging nettle leaves to be submitted to HPE. For extracts with 35% ethanol as solvent, it was observed an increase ($p<0.05$) of about 260% (from 73.98 ± 2.860 to 266.9 ± 6.086 mg_{Trolox}/g DM, when comparing extracts obtained at atmospheric pressure and by HPE, respectively). For the extracts with 70% ethanol, the increase ($p<0.05$) was about 121% (from 114.2 ± 7.629 to 252.9 ± 18.12 mg_{Trolox}/g DM for N0.1/10.6/70 and N200/10.6/70, respectively). For the best of the author's knowledge, this is the first study where this analysis is fully reported for stinging nettle. Nevertheless **Skąpska et al. (2017)** reported a nettle extract (250 mg/L) with an antioxidant capacity of 187.4 ± 50.6 mg_{Trolox}/g, which was used as an additive to incorporate in a fruit drink. This allows to corroborate the fact that stinging nettle has a high antioxidant capacity and when the extraction occurs by high pressure, the extracts present even a greater capacity than when obtained at atmospheric pressure. These results can also be correlated with the individual compounds profile present in the extracts (see **Table 5.3**since the extracts with higher antioxidant activity (N200/10.6/35 and N200/10.6/70) were also the extracts which presented higher concentrations of 5-*p*-coumaroylquinic acid and 5-*O*-caffeoylquinic acid, compounds that were already associated to a high antioxidant power **(Orcic et al., 2014; Xu, Hu and Liu, 2012)**.

Table 5.2. Antioxidant activity measured by ORAC assay and for antihypertensive activity by IACE inhibition assay; genotoxicity (expressed in percentage of DNA degradation inhibition for antioxidant activity and percentage of DNA degradation for pro-oxidant activity) and cytotoxicity (expressed in percentage of cell metabolism inhibition for Caco-2 and HaCat cell lines). Different letters indicate significant differences ($p<0.05$) between extracts for each assay (differences analysed by column)

Identification of extracts		Antioxidant activity (ORAC assay) (mg Trolox / g DM)	Antihypertensive activity (IACE assay) (%)	Genotoxicity*					
				Antioxidant activity (%)		Pro-oxidant activity (%)		Cytotoxicity	
				With $FeCl_3$	Without $FeCl_3$	With $FeCl_3$	Without $FeCl_3$	Caco-2 (metabolism inhibition, %)	HaCat (proliferation inhibition, %)
N200/10.6/0	200 MPa 10.6 min 0% Ethanol	53.74 ± 9.185 d	92.03 ± 0.1361 a	111.4 ± 13.19 a	13.0 ± 3.61 de	16.6 ± 1.30 ab	21.1 ± 18.0 ab	-6.35 ± 1.37 ab	10.88 ± 2.92 ab
N0.1/10.6/0	0.1 MPa 10.6 min 0% Ethanol	50.03 ± 3.518 d	86.17 ± 1.820 a	57.6 ± 6.26 bc	19.5 ± 2.06 cd	42.7 ± 2.24 a	30.6 ± 9.27 ab	5.14 ± 4.25 a	13.63 ± 0.53 a
N500/10/25	500 MPa 10.0 min 25% Ethanol	95.35 ± 10.22 bc	86.82 ± 4.129 a	59.3 ± 6.11 b	7.5 ± 2.25 e	-31.7 ± 18.3 c	-7.08 ± 6.33 c	-30.3 ± 4.14 c	10.83 ± 2.82 ab
N0.1/10/25	0.1 MPa 10.0 min 25% Ethanol	96.23 ± 9.052 bc	70.46 ± 3.954 b	20.2 ± 0.881 c	11.4 ± 2.58 e	36.9 ± 10.5 a	40.1 ± 5.87 a	-5.09 ± 2.60 ab	6.31 ± 2.31 b
N200/10.6/35	200 MPa 10.6 min 35% Ethanol	266.9 ± 6.086 a	82.53 ± 3.702 a	148.6 ± 30.57 a	48.5 ± 2.89 a	-8.77 ± 6.59 bc	13.5 ± 3.87 bc	-25.9 ± 4.16 c	14.12 ± 1.53 a
N0.1/10.6/35	0.1 MPa 10.6 min 35% Ethanol	73.98 ± 2.860 cd	69.75 ± 1.792 b	50.2 ± 4.83 c	34.3 ± 5.74 b	33.0 ± 4.94 a	30.2 ± 5.99 ab	5.24 ± 4.22 a	9.70 ± 3.06 ab
N200/10.6/70	200 MPa 10.6 min 70% Ethanol	252.9 ± 18.12 a	85.45 ± 1.715 a	124.3 ± 28.91 a	22.9 ± 2.30 c	-15.4 ± 12.3 c	6.45 ± 20.34 bc	-26.0 ± 5.01 c	-
N0.1/10.6/70	0.1 MPa 10.6 min 70% Ethanol	114.2 ± 7.629 b	67.66 ± 1.598 b	59.0 ± 5.58 b	23.9 ± 2.85 c	23.7 ± 13.6 a	12.0 ± 7.22 abc	-9.46 ± 5.36 b	-

* Results presented only for the higher concentrations (in mg DM/mL) tested for each extract: Extract 1: 5.06; Extract 2: 5.12; Extract 3: 4.77; Extract 4: 4.87; Extract 5: 4.94; Extract 6: 4.94; Extract 7: 4.72; and Extract 8: 4.71.

Table 5.3. Pearson correlations (R value) and respective p-value in parenthesis. It should be noted that the table is labelled with a colour code (only the significant correlations ($p<0.05$) are highlighted in grey cells, and more intense colour represents the highest correlation values (three levels: light grey: R-value below 0.650; medium grey: R-value from 0.650 to 0.750; dark grey: R-value above 0.750)

	ORAC	IACE	DNA degradation	DNA degradation ($+Fe^{3+}$)	Inhibition of DNA degradation	Inhibition of DNA degradation ($+Fe^{3+}$)	Cytotoxicity	
							CaCo-2	HaCat
Phenolics	-0.091 (0.802)	0.835 (0.001)	-0.366 (0.149)	-0.366 (0.199)	0.590 (0.010)	0.447 (0.063)	0.603 (0.038)	-0.334 (0.380)
ORAC	-	-0.017 (0.955)	-0.190 (0.422)	-0.473 (0.035)	0.498 (0.030)	0.751 (0.000)	-0.025 (0.932)	0.198 (0.497)
Neochlorogenic acid	0.967 (0.000)	0.932 (0.036)	-0.367 (0.178)	-0.137 (0.672)	0.898 (0.036)	0.791 (0.014)	-0.194 (0.591)	0.546 (0.129)
Caftaric acid	0.949 (0.001)	-0.071 (0.867)	-0.547 (0.035)	-0.406 (0.191)	0.849 (0.054)	0.451 (0.080)	-0.084 (0.817)	0.296 (0.439)
5-*p*-Coumaroylquinic acid	0.836 (0.039)	0.003 (0.994)	-0.576 (0.025)	-0.636 (0.026)	0.800 (0.018)	0.641 (0.007)	0.260 (0.467)	0.276 (0.472)
Chlorogenic acid	0.968 (0.000)	0.940 (0.032)	-0.782 (0.045)	-0.659 (0.063)	0.901 (0.035)	0.398 (0.127)	-0.190 (0.599)	0.530 (0.142)
Fertaric acid	0.816 (0.025)	0.950 (0.015)	-0.751 (0.001)	-0.751 (0.005)	0.371 (0.174)	0.722 (0.002)	0.441 (0.202)	0.458 (0.215)
4-*p*-Coumaroylquinic acid	0.752 (0.051)	-0.105 (0.805)	-0.594 (0.020)	-0.688 (0.013)	0.443 (0.099)	0.499 (0.049)	0.823 (0.041)	0.164 (0.674)
2-*O*-Caffeoylmalic acid	0.976 (0.000)	0.788 (0.114)	-0.480 (0.070)	-0.356 (0.257)	0.828 (0.061)	0.508 (0.045)	0.967 (0.015)	0.411 (0.272)
Coumaric acid	-0.475 (0.281)	0.713 (0.047)	-0.887 (0.040)	0.320 (0.311)	-0.320 (0.244)	0.132 (0.626)	0.451 (0.191)	0.223 (0.563)
Rutin	0.962 (0.001)	0.884 (0.026)	-0.375 (0.169)	-0.138 (0.670)	-0.034 (0.903)	0.381 (0.146)	-0.203 (0.573)	0.408 (0.275)
***p*-Coumaroylmalic acid isomer 1**	0.760 (0.143)	0.245 (0.559)	-0.524 (0.045)	-0.545 (0.067)	0.773 (0.001)	0.772 (0.000)	0.760 (0.038)	0.776 (0.014)
***p*-Coumaroylmalic acid isomer 2**	0.487 (0.268)	0.284 (0.496)	-0.402 (0.137)	-0.224 (0.484)	0.669 (0.006)	0.804 (0.000)	0.496 (0.145)	0.514 (0.157)
Isoferulic acid	0.620 (0.138)	0.886 (0.016)	-0.712 (0.003)	-0.736 (0.006)	0.720 (0.014)	0.682 (0.004)	0.403 (0.249)	0.368 (0.329)

5.1.1 Antihypertensive activity

Hypertension can also be called arterial high blood pressure, and it refers to the pressure acting on the walls of the arteries, being the major risk for cardiovascular accidents in the world **(Baradaran, Nasri and Rafieian-Kopaei, 2014)**. For so, hypertension can be characterized by a permanent state of oxidative stress, result of the lack of equilibrium between the generation/destruction of reactive oxygen species. One of the cellular paths to achieve a lowering of blood pressure is to use angiotensin II type I receptor antagonists and angiotensin converting enzyme (ACE) inhibitors, which leads to vasoconstriction relaxation and the release of renal sodium ions. Nevertheless, there are few studies concerning the study of ACE inhibitory activity of plant species, being this the first study reported on stinging nettle.

Similarly to the results obtained for ORAC assay, all the extracts obtained at atmospheric pressure present a high antihypertensive activity, with values above 68% of inhibition for all extracts (**Table 5.2)**. Nevertheless, after submitting the nettle extracts to HPE, a clear improvement of this property was observed, except for aqueous extracts, where no significant difference ($p>0.05$) was observed between the HPE and the controls (**Table 4**). For example, for N200/10.6/70 and N0.1/10.6/70 (70% ethanol, HPE and control, respectively) it was possible to observe an increase ($p<0.05$) of 26.3%, from 67.7 ± 1.60 to 85.5 ± 1.72% ACE inhibition (**Table 4**). These results can be correlated to the content of phenolic compounds and flavonoids described in section 3.1, since it was found a significant ($p<0.05$) Pearson correlation (value of 0.835) (**Table 5**) between the ACE inhibition activity and the concentration of total phenolic compounds from the extracts. It is also noteworthy that the extracts that presented a greater improvement after HPE (70% ethanol) were also the ones who presented higher concentration of flavonoids such as rutin, and phenolic acids, such as chlorogenic acid (see **Table 5.3**). Another important note about extracts presenting high ACE inhibitory activity and the presence of particular phenolic compounds, is the particularly high concentration of fertaric and ferulic acids present in N200/10.6/0, N0.1/10.6/0, N500/10/25, N200/10.6/35 and N200/10.6/70 (with ACE inhibition above 82%); there were also found high Pearson correlation values between these variables (**Table 5.3**). These results may indicate that there is a correlation between these factors still not reported in the literature, since only flavonoids and coumarins from plant material are reported to have hypotensive effects **(Gasparotto Junior et al., 2011;**

Seo et al., 2013). Furthermore, some studies have reported that supplementation of ferulic acid to spontaneously hypertensive rats can have beneficial effects **(Kumar and Pruthi, 2014)**, and a study developed by **Vajic et al. (2018)** reported that periodically supplementation of hypertensive rats with nettle extracts (10-200 mg/kg/day) allowed reducing both systolic and diastolic blood pressure, as well as cardiac index.

5.1.2 DNA antioxidant protection and pro-oxidant activity

The efficiency of the different extracts in preventing DNA damage induced by the presence of H_2O_2 (oxidation) was determined by the DNA assay, using electrophoresis to access the level of denaturation of DNA when in the presence of the extract (maximum concentration of ~5.0 mg DM/mL) (see **Figure 5.1**). Two distinct activities can be evaluated by this assay, the antioxidant activity (measuring the level of inhibition of DNA denaturation) and pro-oxidant activity (measuring the level of DNA denaturation). The addition of $FeCl_3$ to the damaging system composed by H_2O_2 allowed studying the extract effects on a more relevant biological standpoint. The combination of H_2O_2 and $FeCl_3$ leads to a greater damage caused to DNA molecule than using H_2O_2 alone (indicating that H_2O_2, in spite of being considered a reactive oxygen species, has limited DNA damage inducing capabilities on its own) **(Silva et al., 2017)**.

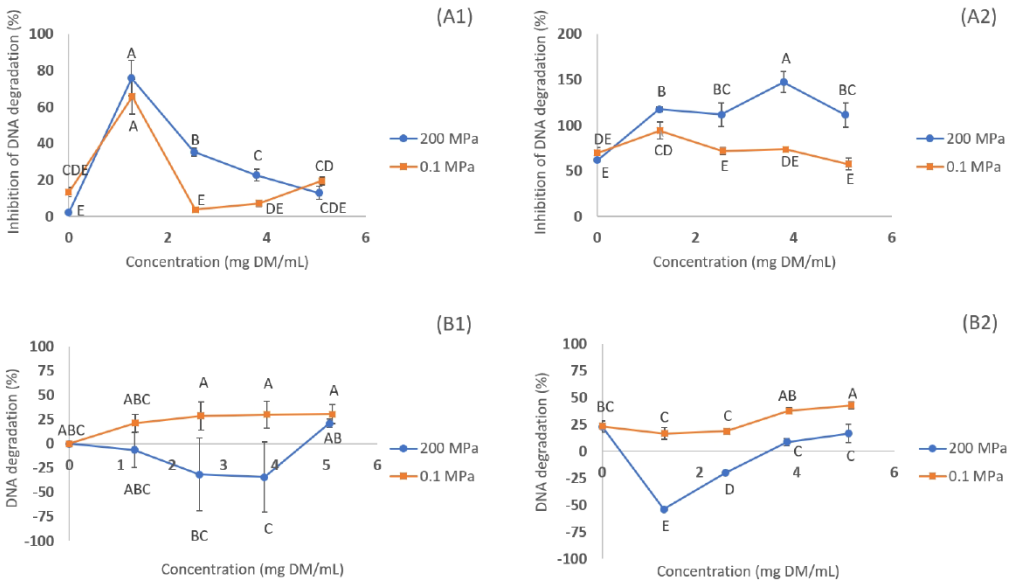

Figure 5.1. Antioxidant activity, *i.e.* prevention of DNA oxidation (by H_2O_2 (A1) or and H_2O_2/$FeCl_3$ system (A2)). Pro-oxidant activity (in the absence (B1) and presence (B2) of iron cations) for aqueous extracts (N200/10.6/0 and N0.1/10.6/0)

When the extracts were added at a concentration of ~5.0 mg DM/mL in the presence of $FeCl_3$, the DNA damage protective effect (antioxidant assay) measured ranged from 59.3 to 148.6% ($p<0.05$), being the order N200/10.6/35 > N200/10.6/70 > N200/10.6/0 > N500/10/25, for the extracts submitted to HPE, while for the controls the higher inhibition of DNA denaturation was 59.0% for the N0.1/10.6/70 (70% ethanol) (**Table 4**). It is noteworthy that, in some cases, the values for inhibition of DNA degradation appear above 100%, indicating a higher fluorescence intensity of the band after exposure to the extract compounds which may indicate the possibility of some interactions between the DNA molecule and the compounds **(Silva et al., 2017)**. Relatively to pro-oxidant assay, the DNA damage in the presence of $FeCl_3$ occurred in a range of -31.7 to 16.6% ($p<0.05$) for the extracts submitted to HPE, while for the controls, the lower value was 23.7% for the N0.1/10.6/70. As can be seen in **Figure 5.1** (B1 and B2) all the tested concentrations of the N200/10.6/0 (submitted to HPE at 200 MPa) presented lower DNA degradation rate than the control (N0.1/10.6/0) using the same concentrations. These results can be correlated to the concentration of individual compounds found in each extract (see **Table 5.3**), since chlorogenic acid and its isomers are known for their DNA-protective activities, and N200/10.6/70 is the richest of all extracts, being the one with higher concentration of chlorogenic acid (5-caffeoylquinic acid) and neochlorogenic acid (3-caffeoylquinic acid) **(Xu, Hu and Liu, 2012)**. This indicate that HPE allows to obtain extracts with improved DNA protective properties, when compared to the controls at 0.1 MPa and with no pro-oxidant activity.

5.1.3 Cytotoxicity

The Caco-2 cell line is a continuous line of heterogeneous human epithelial colorectal adenocarcinoma cells being usually used as a cellular model for the evaluation of the cytotoxic effect of several extracts, since, when confluent, these cells mimic the intestinal epithelium. HaCaT cell line is composed by immortalized human keratinocytes and have been extensively used to study the epidermal homeostasis, being used to access potential biocompatibility of extracts as a cosmetic ingredient. The results in the present work demonstrated that the nettle extracts at a concentration of 1.0 mg DM/mL obtained by HPE did not exert any inhibition of the cellular metabolism of Caco-2 cells, appearing to, in fact, stimulate it, exhibiting negative values for percentage of metabolism inhibition

(ranging from -30.3 to -6.4%) (see **Table 5.2**). When compared to the viability of cells cultured in the absence of extracts (0 mg lyophilized extract/mL – 1.74 ± 0.18% metabolism inhibition), Caco-2 cells cultured in the presence of the different concentrations of HPE extracts demonstrated higher metabolic activity in comparison to the positive control (culture medium and cells), which can be attributed to the supplementation of the culture media with nutrients from the extracts **(Rodrigues et al., 2019**). For HaCat cells, all the extracts at 1.0 mg/mL led to some metabolism inhibition (below 20%), with HPE extracts (N200/10.6/0, N500/10/25 and N200/10.6/35) presenting slightly higher metabolism inhibition values than the controls ones, although not statistically significant ($p>0.05$). These results may indicate that, although not significantly high, stinging nettle extracts could present some antitumoral activity. Nevertheless, more research should be performed to test this hypothesis. In **Table 5.3** it is possible to observe that there is a strong significant ($p<0.05$) correlation between extracts cytotoxicity and the compound 2-*O*-caffeoylmalic acid, while a more moderate correlation appears for the *p*-coumaroylmalic acid isomer 1, indicating that a slight metabolism inhibition for Caco-2 cells occurs probably due to the presence of phenolic compounds in the extracts.

Furthermore, when comparing these results to those of the controls, it can be seen that the presence of extracts obtained at 0.1 MPa led to lower inhibition percentages (with values ranging from -9.5 to 5.2%) ($p>0.05$). Extracts from stinging nettle were already reported as not cytotoxic against HepG2, Hep2c, RD and L2OB cell lines **(Di Sotto et al., 2015; Zekovic et al., 2017a)**.

5.2 Biological activities of extracts obtained from winter savory leaves

5.2.1 Individual compounds

The quantitative compositional analysis of the extracts concerning phenolic compounds content was performed by LC-MS/MS and is presented in **Table 5.4**. The extracts (see **Table 3.1**, **Chapter III**, for nomenclature) with higher total phenolic content (determined by Folin-Ciocalteau method) were S500/20/0 and S348/20/35 (obtained by HPE, with 100% water and 35% ethanol, respectively), with values of 2428.43 ± 224.21 and 2342.93 ± 432.66 mg/100 g DM, respectively. All extracts obtained by HPE are composed by the same type of polyphenols; nevertheless, the control extracts lack some of the compounds, such as tuberonic acid glucoside in control aqueous and ethanolic (70% ethanol) extracts, and sagerinic acid in all control extracts (0, 35, and 70% ethanol). This

indicates that the sagerinic acid, a common phenolic acid present in Lamiaceae family, can be easily extracted by HPE, since it is biologically present inside the cell membrane, which is damaged and destroyed by the HPE process allowing its release **(Rastogi et al., 2007; Stinco et al., 2019).** The extract S348/20/35 was the one with the highest percentage of relevant antioxidants such as rosmarinic acid, salvianolic acid A, and salvianolic acid B isomer. Phenolic compounds are usually distributed in herbal extracts as a function of their own polarity, so phenolic compounds are not generally found in fractions with high amount of ethanol (non-polar fractions), while the most polar compounds remain in the water fraction **(Ćetković et al., 2007b)**. Caffeic acid, rosmarinic acid, salvianolic acid A, and salvianolic acid B isomer were identified in extract S500/20/0, as also tuberonic acid glycoside, butoxyphenol, and sagerinic acid (**Figure 5.2**), which were not identified in extracts S0.1/20/0 and S0.1/1/70 (control extracts).

Winter savory has been studied mainly for its essential oil, however concerning phenolic compounds profile only few studies had been reported. The present work allowed obtaining a much high concentration of caffeic acid (575.40 ± 11.33 mg/100 g DM after an extraction at 500 MPa, 20 min, water), compared to the values reported by **Ćetković et al. (2007b)** of 1.32 and 1.54 of caffeic mg/100 DM g acid for extraction from winter savory dried leaves, using ethyl acetate and *n*-butanol, respectively, for 48 h. Also **Zeljković et al. (2015)** reported the presence of caffeic acid in winter savory dried leaves that after Soxhlet extraction obtained a value of 1.15 and 501.91 mmol/g DM, for chloroform and methanol, respectively. The difference between solvents was attributed to their polarity and consequent capability to extract this phenolic acid. Since chloroform is less polar (polarity of 0.259) compared to methanol (polarity of 0.762), methanol would present a stronger interaction with phenolic compounds, enabling their extraction. The same phenomenon was observed in the present work, where the extracts obtained using water or 35% ethanol presented higher concentrations of phenolic compounds than the 70% ethanolic ones (**Table 5.4**). Concerning the phenolic acid present in higher concentration is all extracts, rosmarinic acid (348.67 ± 88.19 – 2602.74 ± 129.38 mg/100 g), also **Vladimir-Knežević et al. (2014)** had identified it as the major compound present in winter savory leaves, with a value of 3111 mg/100 g DM after a extraction using ethanol in an ultrasonic bath for 30 min.

Table 5.4. Phenolic compounds identified and quantified in winter savory leaves extracts by LC-MS/MS, and antioxidant activity obtained by ORAC assay. Results are shown as mg/100g DM. Different letters indicate significant differences ($p<0.05$) between extracts for each condition set (differences analysed by column)

	Total Phenolic compounds	**Caffeic acid**	**Tuberonic acid glucoside**	**4-Butoxyphenol**	**Rosmarinic acid**	**Sagerinic acid**	**Salvianolic acid A**	**Salvianolic acid B isomer**	**Antioxidant activity (mg_{Trolox}/g DM)**
Formula	-	$C_9H_8O_4$	$C_{18}H_{28}O_9$	$C_{10}H_{14}O_2$	$C_{18}H_{16}O_8$	$C_{36}H_{32}O_{16}$	$C_{26}H_{22}O_{10}$	$C_{36}H_{30}O_{16}$	-
$[M-H]^-$ (m/z experimental)	-	179.0346	387.1654	165.0915	359.0765	719.1608	493.1136	717.1446	-
$[M-H]^-$ (m/z calculated)	-	179.0350	387.1661	165.0921	359.0772	719.1618	493.114	717.1461	-
MS/MS fragments	-	135.0444 (100) 179.0345 (22.6)	387.1656 (100) 207.1023 (35.90) 119.0344 (11.22)	165.0914 (100) 164.0834 (70.80) 149.0596 (36.85) 122.0362 (6.53)	161.0240 (100) 197.0453 (73.67) 179.0343 (24.86)	161.0238 (100) 359.0770 (84.31) 197.0453 (37.66) 179.0343 (4.32)	161.0236 (100) 359.0767 (42.60) 197.0451 (38.47) 135.0443 (32.73)	519.0916 (100) 339.0506 (91.97) 135.0443 (36.14)	-
Identification of extracts									
S500/20/0	2428.43 ± 224.21 a	575.40 ± 11.33 a	227.93 ± 3.50 b	266.35 ± 6.40 a	2020.04 ± 331.42 ab	637.51 ± 16.63 a	1436.40 ± 67.67 b	285.95 ± 0.22 c	404.97 ± 49.35 c
S0.1/20/0	1203.55 ± 864.3 c	530.05 ± 32.49 a	n.d.	245.91 ± 4.29 a	1406.39 ± 38.57 c	n.d.	1348.27 ± 15.68 bc	247.73 ± 2.34 c	404.56 ± 83.04 c
S348/20/35	2342.93 ± 432.66 ab	313.63 ± 4.05 b	263.99 ± 8.77 a	204.81 ± 1.51 b	2602.74 ± 129.38 a	535.82 ± 5.56 b	1635.32 ± 7.63 a	525.26 ± 4.34 a	537.06 ± 62.13 b
S0.1/20/35	1959.28 ± 417.18 ab	306.71 ± 52.45 b	240.44 ± 0.69 b	193.22 ± 10.78 b	1863.22 ± 34.70 bc	n.d.	1599.19 ± 14.69 a	534.43 ± 4.27 a	391.75 ± 21.54 c
S500/1/70	1756.58 ± 191.53 bc	289.78 ± 22.23 b	167.02 ± 6.79 c	132.45 ± 9.68 c	2529.21 ± 34.46 a	28.90 ± 1.64 c	1329.90 ± 38.44 bc	399.87 ± 14.33 b	678.52 ± 67.95 a
S0.1/1/70	1170.73 ± 253.40 c	225.90 ± 9.78 b	n.d.	n.d.	348.67 ± 88.19 d	n.d.	1245.81 ± 23.06 c	364.23 ± 42.05 b	393.05 ± 34.97 c

Note: n.d. means that the compound was not detected.

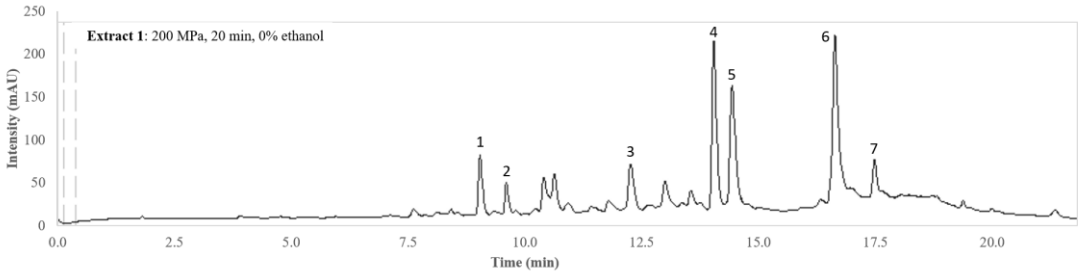

Figure 5.2. LC-MS/MS chromatogram acquired at 320 nm of the analyses of the extract 1 (S500/20/0) from winter savory leaves. The numbers 1 to 7 indicate the seven phenolic compounds identified and quantified in this extract: 1, caffeic acid; 2, tuberonic acid glucoside; 3, 4-butoxyphenol; 4, rosmarinic acid; 5, sagerinic acid; 6, salvianolic acid A; and 7, salvianolic acid B isomer

For the best of the authors knowledge, this is the first study where sagerinic acid and salvianolic acids A and B, common phenolic acids from Lamiaceae family, are reported in winter savory dried leaves extracts.

5.2.2 Antioxidant activity

The oxygen radical absorbance capacity (ORAC) assay is a known method that allows to measure the antioxidant capacity of an extract through the measurement of a fluorescent signal that varies with the reaction between the fluorescein and reactive oxygen species (ROS). The capacity of an extract to act as an antioxidant is evaluated by the capacity to absorb the generated ROS, allowing the fluorescent signal to persist. One of the characteristics that make this method the one with major biologic relevance is the fact that the ROS generator, the AAPH (2,2'-azobis(2-methylpropionamidine) dihydrochloride), is able to produce a peroxyl free radical which is commonly found in the human body; and since the AAPH reacts with both water and lipid soluble substances, it can be used to measure total antioxidant potential. **Table 5.5** shows the antioxidant activity of the various winter savory extracts. Although for the aqueous extracts it was observed no significant differences between the HPE extract (S500/20/0) and the control at atmospheric pressure (S0.1/20/0), it is noteworthy that for the extracts S348/20/35 and S500/1/70, it was observed a clear increase of the antioxidant activity; it was obtained an increase of 37 and 73% compared to the control extracts produced with 35% and 70% ethanol compared with the respective extracts obtained after HPE. Furthermore, the extract S500/1/70 was the one with higher antioxidant capability using ORAC assay (678.52 ± 67.95 mg/g DM), even

though it was one of the extracts presenting lower concentration of individual phenolic compounds, and a relatively low concentration of total phenolic compounds (1756.58 ± 191.53 mg/g DM) when compared, for example, with the extracts S500/20/0 and S348/20/35. These results may be related to the fact that Folin-Ciocalteau method, although being the most used method for total phenolics determination, it is susceptible to some interferants present in the sample, such as proteins and reducing sugars **(Rangel et al., 2013)**. For so, although HPE helps to increase the extraction of components responsible for antioxidant activity, those composites are not only phenolic compounds. For the best of authors knowledge, this is the first study where antioxidant activity by ORAC assay was reported in winter savory leaves extracts

5.2.3 Antimicrobial and antibiofilm activity

All extracts were subjected to antimicrobial screening by initial disk diffusion assays with 10 and 20 mg/mL, which indicated that all the extracts proved to be ineffective against the five bacterial strains tested (Gram-negative: *E. coli* and *S. enteritidis*; Gram-positive: *S. aureus*, *B. cereus*, and *L. monocytogenes*), with no zones of inhibition being observed (data not shown). The MIC-values were determined by observing the lowest concentration of extract that visually inhibited bacterial growth after mixture of the extract with each one of the microorganisms. It was observed a MIC of 20 mg/mL for extract S348/20/35 against *L. monocytogenes* and *S. aureus*, of 20 mg/mL for extract S0.1/20/35 against *S. aureus*, and of 10 mg/mL for extracts S348/20/35, S0.1/20/35, and S500/1/70 against *B. cereus* (**Table 5.5**). For both *E. coli* and *S. enteritidis* there was observed no bacterial growth inhibition at the tested concentrations for any of the extracts studied. These results indicate that the extracts obtained after extraction using 35 or 70% ethanol (controls or HPE ones) are richer in antimicrobial compounds (especially against Gram-positive bacteria) since the aqueous extracts presented no bacterial growth inhibition against any of the five bacterial strains, at the tested concentrations. Contrasting results were reported by **Serrano et al. (2011)** who obtained a MIC-value of 15.10 mg/mL against *E. coli* for ethanol extract from winter savory (obtained by maceration and stirring for 72 h, at room temperature). Additionally, the same authors reported a MIC-value of 3.00 mg/mL for *L. monocytogenes*, indicating that it is needed a much higher concentration of savory

extracts in order to obtain bacterial growth inhibition for Gram-negative than for Gram-positive bacteria **(Serrano et al., 2011)**.

After MIC-values determination, the MBC-values were determined as the lowest concentration of each extract for which bacterial growth was prevented, by drop plate technique. It was only observed an MBC-value of 20 mg/mL for extract S348/20/35 against *L. monocytogenes*, since its initial viability was reduced by at least 99.9% within 24 h. Although other tested strains than *L. monocytogenes* are also Gram-positive, seems that the damage induced by phenolic compounds in the cell wall of *L. monocytogenes* (capable to interfere with the cell wall fluidity, possibly causing its disruption) can lead to a more easy microorganism lysis **(Silva et al., 2013).**

Concerning the biofilm formation inhibition, it can be seen in **Table 5.5** that, generally, all extracts showed a high performance in the inhibition of biofilm formation of all the bacterial strains. Nevertheless, the extracts obtained after HPE presented better results ($p<0.05$) than the control extracts. For both *E. coli* and *B. cereus* strains, all the extracts were able to inhibit biofilm formation, being obtained better results against *B. cereus*, since all extracts were able of >90% biofilm formation inhibition, except for S0.1/1/70 (58.97 ± 8.26%). Furthermore, for *S. aureus* and *L. monocytogenes* the extract S0.1/20/0 (control aqueous extract) presented no activity with the lowest inhibitory effect with values of -3.82 ± 1.66 and 3.76 ± 1.04%, respectively. It was the extract S348/20/35 the one which presented better results, with values of 91.51 ± 0.61, 98.04 ± 1.41, 90.47 ± 1.06, and 96.18 ± 0.67% of biofilm formation inhibition against *E. coli*, *S. aureus*, *B. cereus*, and *L. monocytogenes*, respectively. It is interesting to note that these inhibitory effects were registered for a concentration (20 mg/mL) that was insufficient to completely inhibit microbial growth, being only registered a MIC at this concentration for *L. monocytogenes* and *S. aureus*. These results are in accordance with the reports of several authors that demonstrated that herbal extracts (mostly the ones from Lamiaceae family) rich in phenolic compounds, such as rosmarinic acid, salvaniolic acid A and B, are able of inhibiting both Gram-negative and Gram-positive microorganisms **(Damjanovi et al., 2016; Krapfenbauer et al., 2006; Leahu et al., 2013; Serrano et al., 2011; Silva et al., 2016).**

5.2.4 DNA antioxidant protection and pro-oxidant activity

As discussed in **section 5.2.2** "Antioxidant activity", there are different ROS generating systems, which can significantly affect DNA molecule and lead to its damage and degradation. One of those systems is the mixture of a DNA solution with (1) H_2O_2 and (2) H_2O_2 in the presence of iron cations (addition of $FeCl_3$), in order to evaluate the level of final denaturation of the DNA molecule. The combination of H_2O_2 and $FeCl_3$ leads to an induction of more extensive damage to DNA than using H_2O_2 alone (indicating that H_2O_2, in spite of being considered a ROS generator, has limited DNA damage inducing capabilities on its own). The six different extracts were tested at a maximum concentration of 5.0 mg DM/mL applied to a mixture combining DNA solution and a ROS generating system. From this assay, two biological properties can be obtained, the antioxidant activity (measurement of level of DNA degradation inhibition), and the pro-oxidant activity (measurement of the level of DNA degradation caused by the extract itself) **(Silva et al., 2017)**.

The efficiency of the different extracts in preventing oxidative damage of DNA induced by H_2O_2 (antioxidant activity) was evaluated. In **Table 5.6** it is possible to see that the ability of the several extracts to protect the DNA molecule is dependent on the presence/absence of iron cations in the reaction, since for the higher concentration studied, all extracts evidenced higher DNA oxidation inhibition in the presence of iron, than in its absence. Although it was the extract S500/1/70 the one that presented higher ($p<0.05$) antioxidant activity (124.4 ± 13.31% inhibition of DNA degradation) at the higher concentration (5.0 mg DM/mL), it was the extract S348/20/35 the one with better results ($p<0.05$) at lower concentrations (**Table 5.6, Figure 5.3**), indicating that it is possible to obtain extracts by HPE with high DNA protective effect, even at low concentrations (~1.0 mg DM/mL), assuring to obtain a value of 138.9 ± 5.79 and 84.5 ± 11.38% inhibition of DNA degradation in the absence and presence of $FeCl_3$, respectively. It is interesting to observed that the values of inhibition above 100% indicate that there was a higher fluorescence registered for the DNA band after exposure to the extracts than the band alone; this potentially indicates that some possible interactions may occur between DNA and the compounds present in the extracts as already considered by **Silva et al. (2017)**.

Table 5.5. Minimum inhibitory concentration and biofilm formation inhibition capacity of winter savory leaves extracts against pathogenic bacteria commonly found in food products. Different letters indicate significant differences ($p<0.05$) between extracts for each assay (analysis by column)

	Identification of extracts	***E. coli***	***S. enteritidis***	***S. aureus***	***B. cereus***	***L. monocytogenes***
MIC (mg/mL)	S500/20/0	-	-	-	-	-
	S0.1/20/0	-	-	-	-	-
	S348/20/35	-	-	20	10	20
	S0.1/20/35	-	-	20	10	-
	S500/1/70	-	-	-	10	-
	S0.1/1/70	-	-	-	-	-

		E. coli	***S. enteritidis***	***S. aureus***	***B. cereus***	***L. monocytogenes***
Biofilm formation inhibition (%)	S500/20/0	89.05 ± 0.11 b	81.37 ± 5.05 a	11.75 ± 2.39 d	98.42 ± 1.43 a	89.60 ± 2.68 a
	S0.1/20/0	81.75 ± 1.20 c	56.98 ± 10.26 b	-3.82 ± 1.66 e	96.64 ± 2.35 a	3.76 ± 1.04 c
	S348/20/35	91.51 ± 0.61 b	42.02 ± 9.80 bc	98.04 ± 1.41 a	90.47 ± 1.06 a	96.18 ± 0.67 a
	S0.1/20/35	69.02 ± 3.23 d	12.44 ± 3.22 de	93.51 ± 1.55 ab	89.92 ± 6.73 a	93.05 ± 6.02 a
	S500/1/70	97.62 ± 0.60 a	35.19 ± 6.85 cd	91.03 ± 1.63 b	90.04 ± 1.46 a	93.01 ± 7.05 a
	S0.1/1/70	81.68 ± 1.42 c	5.00 ± 0.51 e	41.16 ± 3.49 c	58.97 ± 8.26 b	74.20 ± 8.31 b

* Results presented only for the higher concentrations (in mg DM/mL) tested for each extract: S500/20/0: 20.10; S0.1/20/0: 20.48; S348/20/35: 19.94; S0.1/20/35: 20.25; S500/1/70: 20.47; S0.1/1/70: 20.08.

Table 5.6. Results for genotoxicity (expressed in percentage of DNA degradation inhibition for antioxidant activity and percentage of DNA degradation for pro-oxidant activity) and cytotoxicity (expressed in percentage of cell metabolism inhibition for HT29-MTX; and in IC_{50} (mg/mL) for Caco-2, TR146, and HeLa cell lines). Different letters indicate significant differences ($p<0.05$) between extracts for each assay (analysis by column)

	Genotoxicity*								
	Antioxidant activity (%)		**Pro-oxidant activity (%)**		**Cytotoxicity**				
	With $FeCl_3$	**Without $FeCl_3$**	**With $FeCl_3$**	**Without $FeCl_3$**	**HT29-MTX (%cell metabolism inhibition)**		**Caco-2**	**TR146**	**HeLa**
					0.5 mg/mL	**1.0 mg/mL**	(IC_{50} (mg/mL))		
S500/20/0	72.8 ± 16.7 bc	65.1 ± 10.6 a	-16.4 ± 13.7 c	-15.5 ± 7.6 d	-25.1 ± 2.0 c	23.9 ± 3.5 d	1.471	1.503	0.629
S0.1/20/0	68.0 ± 4.5 c	35.5 ± 7.2 b	31.2 ± 6.4 b	14.0 ± 3.7 c	13.5 ± 6.6 a	37.7 ± 0.2 a	1.588	1.609	0.671
S348/20/35	99.8 ± 6.7 ab	8.7 ± 2.5 cd	25.3 ± 5.6 b	-47.7 ± 23.3 e	-23.6 ± 4.6 c	29.6 ± 1.7 bc	1.635	0.669	0.846
S0.1/20/35	59.2 ± 2.9 c	6.3 ± 1.6 d	61.5 ± 0.9 a	33.6 ± 4.1 bc	-7.6 ± 1.9 b	38.2 ± 1.9 a	0.756	0.712	1.507
S500/1/70	124.4 ± 13.3 a	21.5 ± 3.4 c	28.0 ± 4.1 b	46.3 ± 5.7 ab	12.8 ± 3.3 a	24.6 ± 0.5 cd	1.932	1.795	1.715
S0.1/1/70	73.3 ± 16.5 c	20.2 ± 4.5 cd	76.2 ± 10.6 a	63.1 ± 12.0 a	22.0 ± 2.0 a	31.9 ± 1.0 b	1.493	1.568	1.530

* Results presented only for the higher concentrations (in mg DM/mL) tested for each extract: S500/20/0: 5.42; S0.1/20/0: 5.39; S348/20/35: 5.45; S0.1/20/35: 5.28; S500/1/70: 5.20; S0.1/1/70: 5.15.

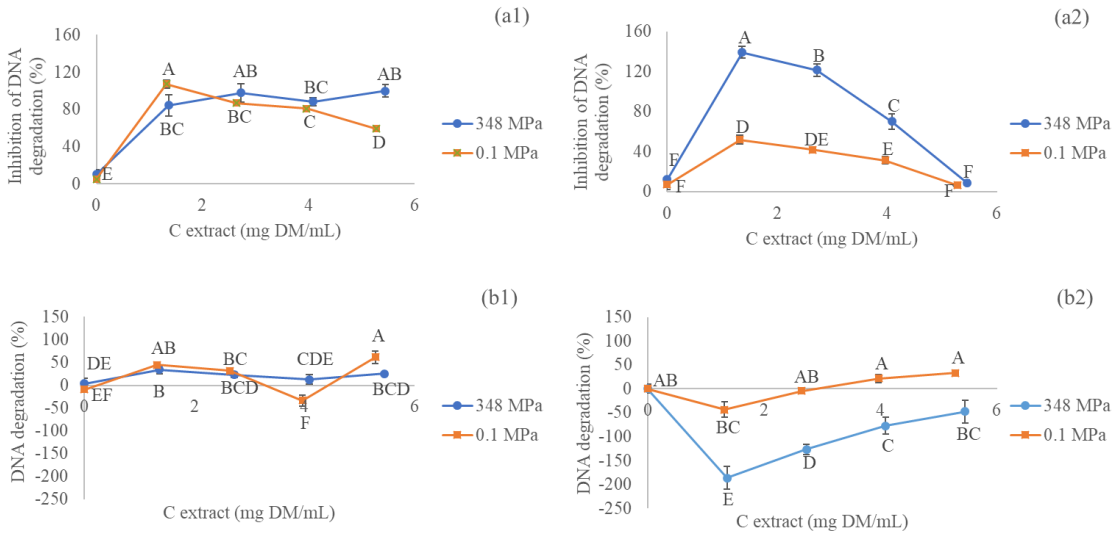

Figure 5.3. Antioxidant activity, i.e. prevention of DNA degradation (in $H_2O_2/FeCl_3$ system (a1), and a H_2O_2 system (a2)). Pro-oxidant activity (in the presence (b1) and absence of iron cations (b2)) for 35% ethanol extracts (extracts S348/20/35 and S0.1/20/35). Different letters indicate significant differences ($p<0.05$) between extracts for each condition set (differences analysed for 348 MPa and 0.1 MPa series individually)

Concerning the pro-oxidant assay (direct DNA damage effect), it is noteworthy that all the control extracts (S0.1/20/0, S0.1/20/35, and S0.1/1/70) presented higher pro-oxidant activity (from 31.2 ± 6.4 to76.2 ± 10.6% of DNA degradation) than the extracts obtained after HPE (S500/20/0, S348/20/35, and S500/1/70), which presented negative values from -16.4 ± 13.7 to 28.0 ± 4.1% of DNA denaturation. These results indicate that in the case of the HPE extracts, there was no relevant interaction between the compounds inducing DNA oxidation, meaning that HPE extracts are able to protect the DNA molecule, with no damaging capacity even at high concentrations. For the best of authors knowledge, this is the first study where antioxidant activity by DNA damage protective effect was reported in winter savory extracts.

5.2.5 Cytotoxicity

The mucus-secreting HT29-MTX intestinal cell line was used to study the possible cytotoxic effect of winter savory extracts using the XTT cell viability assay, by measurement of cell number based on metabolic activity, since, when confluent, these cells mimic human cell behaviour. The results demonstrated that the extracts at 0.5 mg DM/mL did not exert any inhibition of the cellular metabolism, appearing, in fact, to stimulate it

(negative values of metabolism inhibition), which could mean that the presence of the extracts could induce an increase of the metabolic rate of HT29-MTX cell line, and for that demonstrating the absence of cytotoxicity. Only the extract S0.1/1/70 presented an inhibition above 20% (22.0 ± 2.0%), indicating that this extract could cause some inhibition of the cellular development, but also with relevance since values are lower than 30% cell metabolism inhibition. When testing the concentration of 1.0 mg DM/mL, it was observed that all control extracts led to metabolism inhibition above 30% (although very close to the limit **(ISO10993-5, 2009)**, while HPE extracts did not induce significant cell metabolism inhibition of intestinal cells (up to 30%), so demonstrating no cytotoxic effect (**Table 5.6**).

In order to evaluate antitumoral activity, some carcinogenic cell lines were exposed to the extracts. For so, cell lines such as Caco-2 cell line from heterogeneous human epithelial colorectal adenocarcinoma, TR146 cell line from human squamous carcinoma, and HeLa cell line from cervical cancer, were also studied as cellular models to evaluate the cytotoxic effect of extracts. The extracts were tested in a concentration ranging from 0.0002 mg/mL to 2.0 mg/mL, and it was possible to see that the higher concentration, 2.0 mg/mL of all extracts (HPE and controls), led to cell viability values (%) below of 15% for the three cell lines, confirming high inhibitory effect, while lower concentrations (0.2 mg/mL and below) led to cell viability values equal to higher than 100% (**Figure 5.4**). Moreover, extracts exhibited antiproliferative effect on Caco-2, TR146, and HeLa cell lines with IC_{50} values ranging from 0.756-1.932 mg/mL, 0.669-1.795 mg/mL, and 0.629-1.715 mg/mL, respectively. HeLa cell line showed to be the most sensitive one, since lower concentrations of the extracts were needed to inhibit cell survival by 50%. Similar results were obtained by **(Cetojevic-Simin et al., 2004)**, who reported the effect of winter savory extracts pre-treated with 70% methanol on HeLa, HT-29, and MCF-7 cell lines. These authors also observed that HeLa cell line is the most susceptible, with IC_{50} values ranging from 0.41 mg/mL (extract using ethyl acetate) to 0.84 mg/mL (aqueous extracts). Also **Elgndi et al. (2017)** demonstrated that HeLa cells are the most sensitive, obtaining values of IC_{50} ranging from 59.85 to 91.05 µg/mL with extracts obtained by supercritical CO_2 extraction, and reporting that essential oil from winter savory is about 1.5 times more cytotoxic than the extracts obtained by supercritical CO_2 extraction after 4 h.

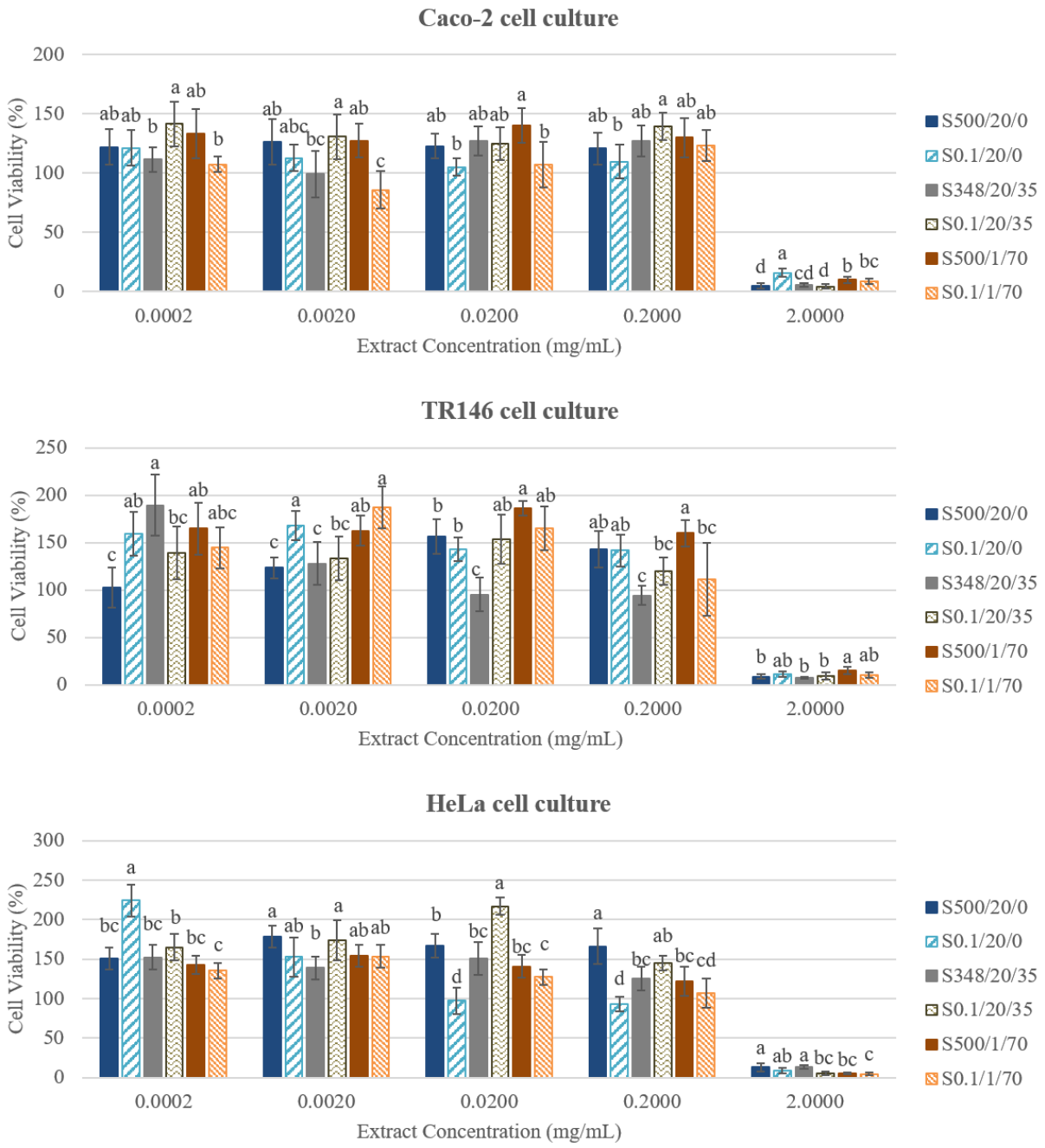

Figure 5.4. Results of cell viability in percentage, obtained for the reaction of the six extracts of *S. montana* with Caco-2, TR146, and HeLa cell culture lines. Different letters indicate significant differences ($p<0.05$) between extracts for each concentration studied (0.0002 to 2.000 mg/mL)

5.2.6 Correlation between variables

A correlation coefficient aims to evaluate the extent to which two variables tend to change together, by describing both the strength and the direction of those relationship. **Table 5.7** shows two different correlations: the Pearson correlation (R-value) that evaluates if the two variables have a linear relationship, *i.e.*, the variables change proportionally to one another; while the Spearman correlation coefficient (rho-value) measures the potential non-linear relationship between two variables, *i.e.*, the variables tend to change together, but not necessarily proportionally.

In **Table 5.7**, it is possible to see that antioxidant activity analysed by ORAC assay has no significant correlation (linear nor non-linear) to any of the other variables under study, which corroborates the hypothesis that, although HPE helps to increase the extraction of components responsible for antioxidant activity, the compounds responsible for the antioxidant activity are probably not only derived from phenolic compounds. Nonetheless, the antioxidant activity measured by DNA assay, presented a high rho-value for total phenolic compounds, as well as for the presence of tuberonic acid glucoside and for the isomer of salvianolic acid B, indicating that the presence of these phenolic compounds may be responsible for the ability of the extracts to be able to protect the DNA molecule against degradation in a system composed by H_2O_2 and iron cations. Also 4-butoxyphenol, sagerinic acid, and salvianolic acid A presented significant ($p<0.05$) strong negative R-values, indicating that as their concentration increases in the extracts, the quantity of damaged DNA decreases. Those phenolic acids were already reported to act as cell protectors against oxidative stress by several authors **(Ma, Tang and Yi, 2019; Mohiseni, 2017; Wu et al., 2009)**.

These results are in accordance to the fact that the extract that presented the highest concentration of those three compounds was the extract S348/20/35, which was also the extract that presented the highest capacity to protect the DNA molecule against ROS activity using a lower extract concentration (**Table 5.6, Figure 5.3**).

Table 5.7. Pearson (R-value) and Spearman (rho-value) correlations and respective p-value in parenthesis. It should be noted that the table is labelled with a colour code (only the significant correlations ($p<0.05$) are highlighted in grey cells, and more intense colour represents the highest correlation values (three levels: light grey: R-value below 0.650; medium grey: R-value from 0.650 to 0.750; dark grey: R-value above 0.750)

			DNA degradation (%)		Inhibition of DNA degradation (%)		Biofilm formation inhibition (%)					Cytotoxicity (% cell viability)			
		ORAC (mg/g)	Without Fe^{3+}	With Fe^{3+}	Without Fe^{3+}	With Fe^{3+}	*E.coli*	*S. enteritidis*	*S. aureus*	*B. cereus*	*L. monocytogens*	HT29-MTX	Caco-2	TR146	HeLa
Pearson Correlation (R)	**Phenolics**	0.376 (0.206)	-0.638 (0.010)	-0.524 (0.045)	-0.431 (0.084)	0.044 (0.862)	0.273 (0.273)	0.307 (0.248)	0.331 (0.179)	0.458 (0.056)	0.656 (0.004)	-0.876 (0.000)	-0.837 (0.022)	-0.806 (0.013)	0.693 (0.016)
	ORAC	-	-0.381 (0.145)	-0.064 (0.813)	-0.267 (0.285)	0.776 (0.000)	0.421 (0.225)	-0.045 (0.908)	0.538 (0.108)	-0.048 (0.896)	0.474 (0.197)	0.382 (0.198)	-0.006 (0.978)	0.117 (0.596)	0.154 (0.433)
	Caffeic acid	-0.200 (0.606)	-0.358 (0.280)	-0.747 (0.021)	0.699 (0.011)	-0.313 (0.321)	0.105 (0.746)	0.887 (0.001)	-0.731 (0.007)	0.680 (0.015)	-0.468 (0.125)	-0.345 (0.273)	0.077 (0.821)	-0.273 (0.446)	-0.756 (0.019)
	Tuberonic acid glucoside	0.532 (0.141)	-0.514 (0.106)	-0.432 (0.245)	-0.635 (0.027)	0.076 (0.814)	0.103 (0.749)	0.082 (0.822)	0.608 (0.036)	0.457 (0.135)	0.744 (0.006)	-0.834 (0.001)	-0.426 (0.192)	-0.205 (0.570)	0.250 (0.517)
	4-Butoxyphenol	0.056 (0.886)	-0.603 (0.050)	-0.742 (0.022)	0.339 (0.281)	-0.301 (0.343)	0.046 (0.888)	0.757 (0.011)	-0.229 (0.473)	0.916 (0.000)	-0.210 (0.512)	-0.647 (0.023)	-0.105 (0.760)	-0.435 (0.209)	-0.718 (0.029)
	Rosmarinic acid	0.542 (0.132)	-0.539 (0.087)	-0.463 (0.209)	-0.283 (0.373)	0.391 (0.208)	0.474 (0.119)	0.212 (0.556)	0.498 (0.099)	0.681 (0.015)	0.414 (0.180)	-0.599 (0.040)	-0.192 (0.571)	-0.714 (0.023)	-0.854 (0.027)
	Sagerinic acid	0.630 (0.069)	-0.885 (0.000)	-0.773 (0.015)	-0.144 (0.656)	0.059 (0.856)	0.426 (0.168)	0.534 (0.112)	-0.094 (0.772)	0.415 (0.179)	0.401 (0.197)	-0.855 (0.000)	-0.758 (0.018)	-0.851 (0.032)	0.590 (0.095)
	Salvianolic acid A	0.495 (0.176)	-0.669 (0.024)	-0.150 (0.699)	-0.518 (0.085)	-0.314 (0.321)	-0.229 (0.474)	-0.080 (0.826)	0.530 (0.076)	0.434 (0.158)	0.400 (0.198)	-0.773 (0.003)	-0.813 (0.027)	-0.274 (0.444)	0.399 (0.287)
	Salvianolic acid B isomer	0.470 (0.202)	-0.210 (0.536)	0.410 (0.285)	-0.883 (0.000)	0.021 (0.948)	-0.238 (0.457)	0.705 (0.023)	0.926 (0.000)	-0.125 (0.699)	0.644 (0.024)	-0.325 (0.303)	-0.811 (0.029)	-0.860 (0.046)	-0.300 (0.432)
Spearman correlation (rho)	**Phenolics**	0.264 (0.384)	-0.579 (0.024)	-0.568 (0.027)	-0.348 (0.171)	0.848 (0.049)	0.375 (0.125)	0.312 (0.239)	0.325 (0.188)	0.349 (0.156)	0.551 (0.022)	-0.862 (0.000)	-0.296 (0.266)	-0.529 (0.043)	0.499 (0.069)
	ORAC	-	-0.412 (0.113)	-0.168 (0.535)	-0.168 (0.505)	0.717 (0.001)	0.491 (0.150)	0.000 (1.000)	0.285 (0.425)	-0.467 (0.174)	0.267 (0.488)	-0.341 (0.255)	0.019 (0.926)	-0.150 (0.494)	0.234 (0.230)
	Caffeic acid	-0.017 (0.966)	-0.736 (0.010)	-0.600 (0.088)	0.441 (0.152)	-0.217 (0.499)	0.172 (0.594)	0.802 (0.005)	-0.455 (0.138)	0.748 (0.005)	-0.063 (0.846)	-0.503 (0.095)	-0.082 (0.811)	-0.418 (0.229)	-0.850 (0.004)
	Tuberonic acid glucoside	0.475 (0.197)	-0.596 (0.053)	-0.322 (0.398)	-0.683 (0.014)	0.100 (0.758)	0.164 (0.611)	-0.062 (0.866)	0.662 (0.019)	0.167 (0.603)	0.812 (0.001)	-0.804 (0.002)	-0.344 (0.300)	-0.181 (0.616)	0.254 (0.509)
	4-Butoxyphenol	0.100 (0.797)	-0.852 (0.001)	-0.733 (0.025)	0.438 (0.155)	-0.228 (0.477)	0.132 (0.684)	0.839 (0.002)	-0.445 (0.147)	0.858 (0.000)	-0.060 (0.854)	-0.602 (0.038)	-0.173 (0.611)	-0.474 (0.166)	-0.833 (0.005)
	Rosmarinic acid	0.550 (0.125)	-0.536 (0.089)	-0.417 (0.265)	-0.420 (0.175)	0.538 (0.071)	0.718 (0.009)	-0.109 (0.763)	0.510 (0.040)	0.084 (0.795)	0.685 (0.014)	-0.552 (0.063)	-0.227 (0.502)	-0.467 (0.174)	-0.100 (0.798)
	Sagerinic acid	0.566 (0.112)	-0.560 (0.073)	-0.836 (0.005)	0.872 (0.042)	0.571 (0.052)	0.692 (0.013)	0.458 (0.183)	-0.041 (0.899)	0.362 (0.248)	0.515 (0.047)	-0.728 (0.007)	0.124 (0.717)	-0.343 (0.332)	0.279 (0.468)
	Salvianolic acid A	0.417 (0.265)	-0.800 (0.003)	-0.300 (0.433)	-0.497 (0.101)	-0.091 (0.779)	0.067 (0.837)	0.109 (0.763)	0.448 (0.145)	0.399 (0.199)	0.615 (0.033)	-0.804 (0.002)	-0.464 (0.151)	-0.333 (0.347)	-0.650 (0.048)
	Salvianolic acid B isomer	0.167 (0.668)	-0.045 (0.894)	0.250 (0.516)	-0.916 (0.000)	0.042 (0.897)	-0.119 (0.712)	0.742 (0.014)	0.958 (0.000)	-0.427 (0.167)	0.573 (0.051)	-0.266 (0.404)	-0.436 (0.180)	-0.079 (0.829)	-0.833 (0.038)

Relatively to biofilm formation inhibition, it was found significant rho-values for rosmarinic acid and sagerinic acid against *E. coli*, while caffeic acid seems to be more related with the biofilm formation inhibition for *S. enteritidis* and *B. cereus*, presenting strong significant R-values. It is interesting to note that for *S. aureus* biofilm formation inhibition, the R-value obtained for caffeic acid, although statistically significative ($p<0.05$), is negative (-0.731), indicating that as the concentrating of caffeic acid increases in the extracts, seems to lose the capability to inhibit biofilm formation for *S. aureus*. This result is in accordance with the percentage of biofilm formation inhibition (11.75 ± 2.39 and -3.82 ± 1.66) obtained for S500/20/0 and S0.1/20/0, the extracts with higher concentration of caffeic acid. This effect was already partially observed by **Kępa et al. (2018)**, who reported that *S. aureus* (isolated from intractable wound infections) can be susceptible to caffeic acid alone, but the combination of the phenolic acid with antibiotics, or other phenolic compounds, seems to be more effective. Nevertheless, a strong significant correlation (R-value) was found between biofilm formation inhibition for *S. aureus* and the presence of salvianolic acid B isomer, as well between biofilm formation inhibition for *B. cereus* and 4-butoxyphenol.

Concerning the cytotoxicity of the extracts and the possible responsible compounds, in **Table 5.7** it is observed a strong significant ($p<0.05$) negative correlation (R-values) between cytotoxicity and the compounds tuberonic acid glycoside, sagerinic acid, salvianolic acids A and B, while a more moderate negative correlation (R-value) appears for 4-butoxyphenol and rosmarinic acid, indicating that metabolism inhibition and consequent decrease of cell viability for all the cancerous cell lines occurs probably due to the presence of phenolic compounds in the extracts, indicating a possible antitumoral activity.

5.3 Conclusions

The main results of the present chapter indicate that both stinging nettle and winter savory leaves extracts have relevant biological activities, such as antioxidant, DNA protective, and potential as antiproliferative. For stinging nettle extracts, a clear improvement of all biological activities of the extracts was observed when the extracts where obtained by high pressure assisted extraction. The extracts obtained at 200 MPa, 10 min, 35 and 70% ethanol, were the ones which presented higher concentrations of phenolic

acids (5-*O*-caffeoylquinic acid, fertaric and isoferulic acid) and flavonoids (rutin), being these compounds related to the highest antioxidant, DNA-protective, and antihypertensive activities. These results scientifically validate the ancient application of stinging nettle in traditional medicine and demonstrate that emerging technology may be used to obtain more active extracts and safer (either in food or cosmetic applications).

Concerning winter savory extracts, the extract S348/20/35, obtained at 348 MPa, 20 min, using 35% ethanol that showed to be the extract with higher concentration of individual phenolic compounds, higher potential as antioxidant, DNA protection, with higher ability to inhibit biofilm formation of *E.coli*, *S. aureus*, *B. cereus*, and *L. monocytogenes*, and was also the only extract that showed a value of MBC of 20 mg/mL against *L. monocytogenes*. The extracts obtained by HPE also presented a higher antioxidant activity than controls, either measured by ORAC assay or by DNA degradation assay, both biological important methods. It is noteworthy that winter savory extracts, especially the ones obtained after HPE, were able not only to not induce significative damage in the DNA molecules compared to the controls, as were also able to protect it against the damage caused by oxidative stress in the presence of ROS. Concerning the cytotoxicity of the extracts, it was observed that HPE extracts, in a concentration of 0.5 mg/mL, were not considered harmful to HT29 cell lines (a cell culture commonly used to assess the biocompatibility of extracts to human intestinal cells). Nevertheless, in a higher concentration (>1.0 mg/mL), although HPE extracts do not reveal significant inhibition, the control extracts demonstrate slight significant reduction of the cell viability. The activity of extracts upon a set of cancerous cell lines, indicated that these extracts may present a potential antitumoral activity.

It can be concluded that all the extracts biological activities are probably caused by phenolic components. These bioactive extracts require *in vivo* studies to validate the positive *in vitro* properties here explored, and the strong antioxidant activity open the need for further research concerning other biological activities still yet to discover, such as the antitumoral potential.

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CHAPTER VI

RESULTS AND DISCUSSION | CASE-STUDY

Adapted from:

Moreira, S.A. et al (2020) - Effect of winter savory leaves extract obtained by high hydrostatic pressure on quality of carrot juice.

Summary

6 Overview

This chapter aims to collect the data concerning the analysis of the case-study: fortification of carrot juice with winter savory extract (selection of results from **Chapter IV**, **Section 4.2**; and **Chapter V**, **Section 5.2**).

The consumption of vegetable juices has increased due to their characteristics such as freshness/natural, high nutritional value, and low calories. Beverages are a convenient way of consuming bioactive compounds, being carrot juice one of the most popular juices. High hydrostatic pressure, which has been mainly used to replace thermal processing, is now also being successfully applied to recover bioactive compounds from herbs as extraction technology. The aim of this work was to determine the effect of addition of winter savory extract obtained by HPE in raw carrot juice subjected to HPP treatment.

6.1 General aspects

Carrot juice was selected as the food product to be studied since it has a relatively lower antioxidant potential than other vegetable juices **(Wootton-Beard, Moran and Ryan, 2011)** it is highly prone to growth of both spoilage and pathogenic microorganisms due to its high pH (6.0-6.5) and high water activity (aw > 0.85). Also, since its pH is close to neutral (**Table 6.1**), it should not majorly affect the biological activities of the bioactive compounds present in the winter savory extract, since also the extracts have a pH ranging from 6.0 to 6.5 (**Appendix C5**). Due to these limitations, carrot juice must be consumed refrigerated, and no more than 1-2 days after production, fact that greatly affects its marketability **(Pilavtepe-Çelik, 2013)**. For so, high pressure processing was used as a non-thermal pasteurization technology in order to improve the juice microbiological stability (with or without addition of winter savory extract), without the use of high temperatures, which could destroy nutrients, bioactive compounds, or even affect the juice organoleptic properties, such as colour and taste.

Winter savory leaves extract obtained by HPE at 500 MPa, 20 min, using water as solvent, were used due to its high antioxidant activity already demonstrated (**Chapter IV**, **Section 4.2**; and **Chapter V**, **Section 5.2**). Since it was an extract rich in total phenolic compounds, such as caffeic acid, rosmarinic acid, sagerinic acid, and salvianolic acid A, this extract presented diverse biological activities, such as high antioxidant activity reported for ABTS, DPPH, FRAP, and ORAC assays, as also for DNA degradation inhibition; it also presented anti-biofilm formation against five bacterial strains, and no pro-oxidant activity for DNA assay (no DNA degradation) **(Chapter IV, Section 4.2; and Chapter V, Section 5.2)**. The concentration of extract in the final juice, 1.0 $mg_{DM}/mL_{solution}$, was selected as reported by other studies which supplemented different food products with herbal extracts **(Adiamo et al., 2017; Skąpska et al., 2017; Trigo et al., 2018)**.

6.2 Microbiological analyses

The evaluation of microbial safety of a food product is essential for its consumption and merchantability. Three groups of spoilage microorganisms, total aerobic mesophilic bacteria (TAM), Enterobacteriaceae (ENT), and yeasts and moulds (YM), were monitored throughout 15 days under refrigeration.

Table 6.1. pH-values, colour parameters, phenolics and flavonoids content found in carrot juice samples, with and without addition of winter savory extracts. Different letters indicate significant differences ($p<0.05$) between samples for each assay (differences analysed by column)

		pH	Colour parameters				Total phenolic compounds (mg_{GAE}/ mL)	Total flavonoids (mg_{Rutin}/ mL)
			*a**	*b**	*L**	ΔE*		
Initial (t0)	**Juice**	6.49 ± 0.01 abc	14.66 ± 3.00 bc	21.67 ± 8.20 ab	35.56 ± 6.07 ab	-	71.77 ± 7.18 ab	155.09 ± 0.17 d
	Juice + HPP	6.50 ± 0.00 ab	14.42 ± 2.69 c	21.78 ± 7.60 ab	36.03 ± 5.79 ab	9.66 ± 0.42 bcd	76.95 ± 4.79 ab	165.58 ± 2.44 cd
	Juice + Extract	6.52 ± 0.01 ab	15.18 ± 2.85 bc	21.82 ± 7.68 ab	35.34 ± 5.80 b	9.81 ± 0.48 bcd	92.90 ± 4.13 ab	172.00 ± 8.38 bcd
	Juice + Extract + HPP	6.53 ± 0.00 a	15.34 ± 2.74 bc	21.55 ± 6.95 ab	35.34 ± 5.59 b	9.14 ± 0.46 d	97.01 ± 12.98 a	177.93 ± 1.05 bc
Storage 5 days (t5)	**Juice**	6.45 ± 0.00 abcd	16.99 ± 2.67 ab	25.19 ± 7.65 ab	38.73 ± 4.95 ab	10.54 ± 0.52 bc	68.39 ± 10.95 ab	167.68 ± 0.17 cd
	Juice + HPP	6.51 ± 0.03 ab	14.53 ± 1.78 c	18.98 ± 4.62 b	38.07 ± 3.65 ab	7.22 ± 0.33 e	83.42 ± 3.77 ab	166.07 ± 6.29 cd
	Juice + Extract	6.37 ± 0.01 cd	14.27 ± 1.57 c	21.02 ± 4.12 ab	37.40 ± 3.41 ab	6.42 ± 0.37 e	96.39 ± 0.36 a	179.65 ± 1.05 abc
	Juice + Extract + HPP	6.50 ± 0.00 ab	13.99 ± 2.01 c	19.13 ± 4.41 b	35.95 ± 4.14 ab	7.04 ± 0.39 e	94.85 ± 3.84 a	196.20 ± 0.35 a
Storage 15 days (t51)	**Juice**	3.95 ± 0.02 f	19.01 ± 2.90 a	27.20 ± 8.33 a	40.53 ± 5.00 a	12.87 ± 0.11 a	60.54 ± 11.75 b	182.00 ± 5.06 abc
	Juice + HPP	6.39 ± 0.04 bcd	14.11 ± 2.73 c	20.93 ± 7.18 ab	35.91 ± 5.73 ab	9.45 ± 0.33 cd	73.11 ± 1.09 ab	187.80 ± 9.43 ab
	Juice + Extract	4.95 ± 0.05 e	17.04 ± 2.72 ab	25.36 ± 7.54 ab	39.08 ± 5.06 ab	10.74 ± 0.36 b	86.08 ± 0.73 ab	177.93 ± 2.10 bc
	Juice + Extract + HPP	6.35 ± 0.07 d	13.86 ± 2.77 c	20.42 ± 6.95 b	35.05 ± 5.75 b	9.31 ± 0.24 d	94.83 ± 16.72 a	177.19 ± 2.79 bc

Four samples were studied (see **Table 3.1**, **Chapter III**, for nomenclature)), raw juice used as control ('juice'), raw juice submitted to high pressure pasteurization ('juice+HPP'), raw juice supplemented with extract ('juice+extract') and raw juice supplemented with extract and submitted to high pressure pasteurization ('juice+extract+HPP').

As shown in **Figure 6.1**, the initial microbiological loads of raw initial juice were 4.95 ± 0.02, 6.13 ± 0.05, and above 4.00 log CFU/mL for ENT, TAM, and YM, respectively. These values are according to those reported by **Stinco et al. (2019)** for carrot juice before high pressure processing that also presented viable cell counts of 6.00 and 4.11 log CFU/mL for TAM and YM, respectively.

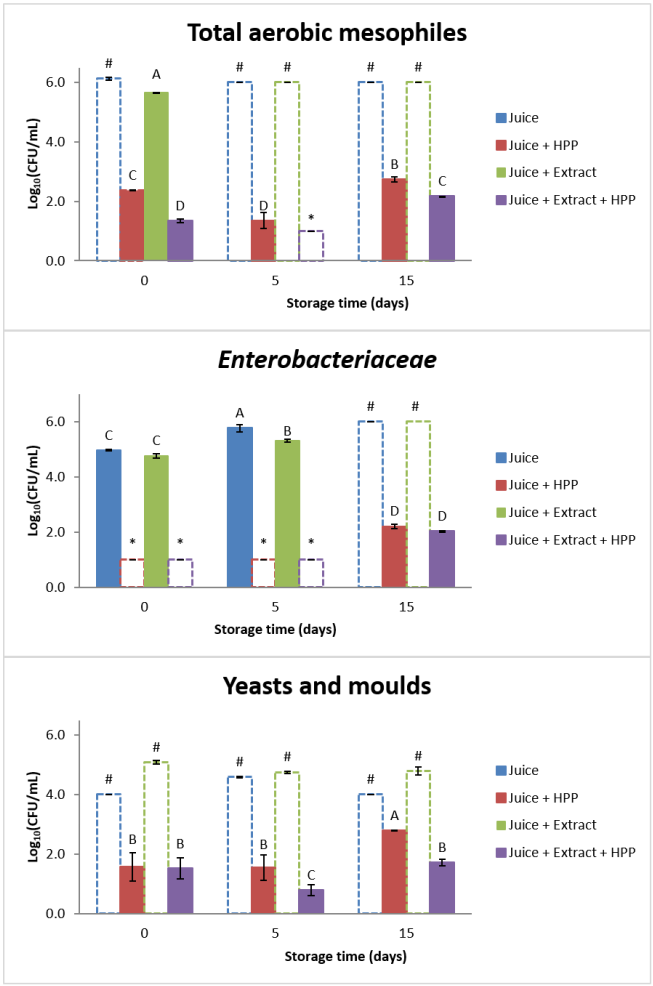

Figure 6.1. Microbial counts (log CFU/mL) of TAM, ENT and YM in the four different samples of juice during storage for 15 days under refrigeration (4ºC). Different letters indicate significant differences ($p<0.05$) between conditions. Values shown as 6 and 4 (unfilled bars marked with #) and 1 (unfilled bars marked with *) log units, mean values above (higher than 6 log CFU/mL for TAM and ENT and 4 log CFU/mL for YM), and below (lower than 1 log CFU/mL for TAM and ENT) the quantification and detection limits, respectively

The four samples presented different behaviours throughout the 15 days storage. The two samples submitted to high pressure pasteurization ('juice+HPP' and 'juice+extract+HPP') presented microbial counts significantly lower ($p<0.05$) than the two other samples ('juice' and 'juice+extract') not reaching the maximum limit (of 6.00 log CFU/mL for TAM and ENT and 4.00 log CFU/mL for YM) during the 15 days of storage, as expected, since high pressure processing leads to microbial inactivation of the three groups of microorganisms studied **(Considine et al., 2008)**. When comparing the juices with and without supplementation of extract, it was observed an interesting behaviour, since the 'juice+HPP' and the 'juice+extract+HPP' had significantly ($p<0.05$) different TAM microbial counts, since the 'juice+extract+HPP' presented lower microbial counts (1.34 ± 0.06, <1.00, and 2.16 ± 0.01 log CFU/mL for 0, 5, and 15 days of storage) than the 'juice+HPP' (2.37 ± 0.02, 1.35 ± 0.27, and 2.74 ± 0.08 log CFU/mL for 0, 5, and 15 days of storage) (**Figure 6.1**). This effect may be a consequence of the antimicrobial activity reported for winter savory extracts in **Chapter V**, **Section 5.2**. A similar behaviour was observed for YM, with lower microbial loads obtained for the 'juice+extract+HPP' compared to the other samples (**Figure 6.1**). Concerning ENT counts, the potential antimicrobial effect could be observed at the 5th day of storage, where the 'juice' and 'juice+extract', *i.e.*, without high pressure pasteurization, presented different ($p<0.05$) results (5.75 ± 0.14 and 5.31 ± 0.06 log CFU/mL, respectively); nevertheless, those samples reached the maximum limit between the 5th and the 15th day of storage, while the samples submitted to high pressure processing presented low microbial counts and similar ($p>0.05$) in value (~2.0 log CFU/mL). Thus, this study demonstrated that storage of carrot juice supplemented with an extract from winter savory leaves and processed by high pressure pasteurization, shows a clear longer ($p<0.05$) storage microbial stability, compared to the control juices ('juice' and 'juice+HPP'), which may suggest a possibly synergy between the supplementation of a beverage with an herbal extract and high pressure processing.

6.3 Physicochemical analyses

6.3.1 pH

The initial pH of raw carrot juice was 6.49 ± 0.01, which is according to the values reported in literature **(Koutchma et al., 2016; Stinco et al., 2019; Trigo et al., 2018)**. As

can be seen in **Table 6.1**, pH did not change throughout storage time, nor due to the treatment of carrot juice (submission to high pressure treatment nor the addition of extract), being only observed two significantly ($p<0.05$) lower values for 'juice' and 'juice+extract' of 3.95 ± 0.02 and 4.95 ± 0.05, respectively, after 15 days of storage. These results can be explained by the metabolism of the spoilage microbiota (that was found at higher levels in these two samples) observed for these two samples that can produce organic acids. Contrarily, both juices submitted to high pressure ('juice+HPP' and 'juice+extract+HPP') maintained the pH-values stable ($p<0.05$) throughout the 15 days of storage, ranging from 6.35 ± 0.07 to 6.53 ± 0.00 (**Table 6.1**).

6.3.2 Colour

Juice colour is an important parameter for the consumer, being essential its preservation along storage. In **Table 6.1** it is possible to see that L^* (lightness), a^* (red/green colour), and b^* (yellow/blue colour) parameters did not significantly ($p>0.05$) change during the 15 days storage. Since a^* and b^* values ranged from 13.86 ± 2.77 to 19.01 ± 2.90 CIELAB units and 18.98 ± 4.62 to 27.20 ± 8.33 CIELAB units, all the juices remained in the red/yellow area, while the L^* ranging from 35.05 ± 5.75 to 40.53 ± 5.00 CIELAB units indicate that the juices were darker than bright (similarly to results in the literature **(Leahu et al., 2013)**). These results show that, in general, all the juices presented a darkish-orange colour, indicating that the supplementation with herbal extract did not significantly ($p<0.05$) affect the final juice colour. The main compounds responsible for carrot juice colour are carotenoids and anthocyanins, and data in literature suggested that HPP may induce colour modifications as result of breakage of carotenoid-protein complexes, which could lead to a better dispersion of carotenoids and a more dark-coloured juice. This hypothesis is supported by the fact that the two samples submitted to high pressure processing presented lower L^* values (35.91 ± 5.73 and 35.05 ± 5.75 CIELAB units for 'juice+HPP' and 'juice+extract+HPP', respectively) than the samples without HPP treatment (40.53 ± 5.00 and 39.08 ± 5.06 CIELAB units for 'juice' and 'juice+extract', respectively) after 15 days. Nevertheless, further research is needed to link that premise to the results. Concerning the total colour difference (ΔE^*) between the raw initial juice and the other samples, the values ranged from 6.42 ± 0.37 to 12.87 ± 0.11 CIELAB units (**Table 6.1, Figure 6.2**), indicating that a trained consumer could perceive

the colour differences between the different juices, since **Krapfenbauer et al. (2006)** defined that a ΔE* higher than 3.5 CIELAB units indicates a noticeable difference between food products that can be perceptible by a trained consumer. Nonetheless, the ΔE* value obtained for 'juice+HPP', 'juice+extract', and 'juice+extract+HPP' (7.22 ± 0.33, 6.42 ± 0.37, and 7.04 ± 0.39 CIELAB units, respectively) was significantly lower ($p<0.05$) than the ΔE* value for raw carrot juice (10.54 ± 0.52 CIELAB units) after 5 days of storage, indicating that high pressure processing and the supplementation with extract can effectively reduce the total colour change of carrot juice during refrigerated storage.

Beginning of storage (t0) **After 5 days of storage (t5)** **After 15 days of storage (t15)**

Figure 6.2. Photographs of the four juice sample throughout storage time. From left to right: Sample A, 'raw juice'; sample B, 'juice+HPP', sample C, 'juice+extract'; and sample D, 'juice+extract+HPP'

6.4. Total phenolic compounds and flavonoid content

In order to compare the influence of the addition of herbal extract to the carrot juice on its concentration of bioactive compounds, total phenolics and total flavonoids were studied. As can be seen in **Table 6.1**, no significant differences were observed for total phenolic compounds content among all four carrot juice samples (with/without high pressure processing; with/without supplementation with extract). Nevertheless, it was observed an increase of total phenolics from the initial value (71.77 ± 7.18 mg_{GAE}/mL) to the 'juice+extract' (92.90 ± 4.13 mg_{GAE}/mL) and 'juice+extract+HPP' (97.01 ± 12.98 mg_{GAE}/mL) on the day the mixture as performed. These results were higher than the ones reported by **Wootton-Beard, Moran and Ryan (2011)** who obtained a total phenolics concentration of 0.45-0.61 $mg_{ferulic\ acid\ equivalents}$/mL from two commercial carrot juices. The

large difference observed between the values from the two studies may be because the commercial juices analysed in the last work were thermally treated, which could lead to thermo-sensitive compounds degradation. Anyway, care must be taken when posing this hypothesis, since total phenolics concentration in the work developed by **Wootton-Beard, Moran and Ryan (2011)** and those in the present work, are expressed using different standard compounds, ferulic acid in the former and gallic acid in the latter, even if the values differ by more than one order of magnitude.

Concerning total flavonoids content, a similar behaviour was observed, since the initial juice (day zero) had a value of 155.09 ± 0.17 mg_{Rutin}/mL, while the 'juice+extract' and 'juice+extract+HPP' presented values of 172.00 ± 8.38 mg_{Rutin}/mL and 177.93 ± 1.05 mg_{Rutin}/mL, respectively. These differences remained until the end of the 15 days of study, where the juices supplemented with extract presented higher concentrations of phenolic compounds and total flavonoids. It is noteworthy that, generally, both juices submitted to high pressure processing ('juice+HPP' and 'juice+extract+HPP') presented higher values than its counterparts ('juice' and 'juice+extract'). These results indicate that high pressure might lead to an easier and so enhanced phenolic compounds and total flavonoids extraction.

6.5. Antioxidant activity

Antioxidant activity from food products can be accessed by different methods, which mostly rely on single electron transfer or hydrogen atom transfer. Methods such as reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) are simple, cost effective, easily interpreted and display either reduction capacity (FRAP) or direct free radical inhibition (DPPH and ABTS) **(Thaipong et al., 2006; Wootton-Beard, Moran and Ryan, 2011)**.

The antioxidant activity values obtained for the different assays presented high variability from one another; since while for FRAP assay the values ranged from 372.87 ± 6.13 to 601.87 ± 22.63 mg_{AIS}/mL, it were obtained values ranging from 57.38 ± 1.89 to 145.17 ± 0.71 mg_{Trolox}/mL and 112.03 ± 13.50 to 173.58 ± 1.46 mg_{Trolox}/mL, for ABTS and DPPH assays, respectively (**Table 6.2**). A similar behaviour was reported by **Wootton-Beard, Moran and Ryan (2011)**, who also observed higher values of antioxidant activity for FRAP assay, followed by DPPH and ABTS methods. These differences can be

attributed to the fact that ABTS radical is soluble in both water and lipophilic solvents, DPPH radical is better soluble in organic solvents, and FRAP measures the reducing potential of an antioxidant reacting with a ferric complex, occurring mostly at an acidic and not neutral pH **(Sekhon-Loodu and Rupasinghe, 2019)**. It is interesting to notice that the juices submitted to high pressure processing presented higher antioxidant activities than the juices that were just refrigerated.

Table 6.2. Results for antioxidant activity (by ABTS, DPPH, and FRAP assays) of the juice with and without addition of winter savory extracts. Different letters indicate significant differences ($p<0.05$) between samples for each assay (analysis by column)

		ABTS assay (mg_{Trolox}/mL)	DPPH assay (mg_{Trolox}/mL)	FRAP assay (mg_{AIS}/ mL)
Initial (t0)	**Juice**	103.76 ± 0.00 d	149.42 ± 8.30 bcd	390.87 ± 30.01 cd
	Juice + HPP	116.97 ± 4.81 cd	172.66 ± 0.81a	393.64 ± 10.69 cd
	Juice + Extract	134.36 ± 0.87 ab	173.58 ± 1.46 a	454.53 ± 10.69 bc
	Juice + Extract + HPP	145.17 ± 0.71 a	165.53 ± 2.93 ab	490.87 ± 26.24 b
Storage 5 days (t5)	**Juice**	114.46 ± 3.15 cd	112.03 ± 13.50 e	372.87 ± 6.13 d
	Juice + HPP	114.74 ± 3.07 cd	133.89 ± 1.30 de	464.87 ± 16.18 b
	Juice + Extract	136.26 ± 3.86 ab	140.51 ± 4.96 cd	440.09 ± 14.77 bc
	Juice + Extract + HPP	136.26 ± 2.13 ab	156.67 ± 3.25 abc	601.87 ± 22.63 a
Storage 15 days (t15)	**Juice**	57.38 ± 1.89 e	136.59 ± 2.20 cd	373.31 ± 2.36 d
	Juice + HPP	103.81 ± 1.50 d	127.16 ± 9.03 de	573.87 ± 1.26 a
	Juice + Extract	68.42 ± 8.83 e	131.65 ± 0.24 de	437.76 ± 6.44 bcd
	Juice + Extract + HPP	128.34 ± 5.75 bc	158.31 ± 0.85 abc	589.26 ± 19.25 a

Similar to the concentration of total phenolic compounds and total flavonoids, also the juices with supplementation of extract ('juice+extract' and 'juice+extract+HPP') presented higher values of antioxidant activity for ABTS, DPPH, and FRAP assays (**Table 6.2**). This was expected since the general consensus is that the antioxidant property of many

vegetables and herbs is directly related to the presence of specific phenolic compounds (**Table 6.3)**.

Table 6.3. Pearson (R-value) and Spearman (rho-value) correlations and respective p-value in parenthesis. It should be noted that the table is labelled with a colour code (only the significant correlations ($p<0.05$) are highlighted in grey cells, and more intense colour represents the highest correlation values (three levels: light grey: R-value below 0.650; medium grey: R-value from 0.650 to 0.750; dark grey: R-value above 0.750)

		Flavonoids	ABTS	DPPH	FRAP
Pearson correlations (R-value)	**Phenolics**	0.681 (0.018)	0.854 (0.001)	0.702 (0.012)	0.704 (0.012)
	Flavonoids	-	0.049 (0.820)	0.759 (0.066)	0.827 (0.001)
	ABTS	-	-	0.481 (0.017)	0.431 (0.035)
	DPPH	-	-	-	0.691 (0.037)
	FRAP	-	-	-	-
Spearman correlations (rho-value)	**Phenolics**	0.249 (0.241)	0.756 (0.000)	0.445 (0.029)	0.603 (0.002)
	Flavonoids	-	0.121 (0.572)	-0.165 (0.440)	0.674 (0.019)
	ABTS	-	-	0.602 (0.002)	0.518 (0.010)
	DPPH	-	-	-	0.210 (0.324)
	FRAP	-	-	-	-

6.6. Conclusions

The present work aimed to evaluate the supplementation of carrot juice with winter savory leaves extract, and its effect on final juice quality characteristics, such as pH, colour, bioactive compounds concentration and antioxidant activity. For so, the extract was added to raw carrot juice (1.0 mg extract/mL juice), which was then submitted to high pressure pasteurization and stored at 4 °C for 15 days. Supplemented juices allowed to obtain lower microbial counts than the non-supplemented ones; and even though the high pressure pasteurization seemed to have the major effect on microbial inactivation, it were observed lower counts for the juice supplemented with extract and submitted to high

pressure. Generally, the supplementation of the juice with extract did not affect ($p>0.05$) pH nor the colour parameters. Concerning the total phenolic compounds and total flavonoids, as well as antioxidant activity (assessed by ABTS, DPPH, and FRAP methods), the values were generally higher ($p<0.05$) in supplemented juices during the 15 days of storage. These data suggest that the addition of winter savory leaves extract (1.0 mg/mL) in carrot juice treated with HPP can effectively improve microbial safety throughout refrigerated storage as well as antioxidant activity, without risking other characteristics of the juice, such as the colour or the acidity. In further studies it would be interesting to study the effect of *in vitro* digestion of the juice to understand the stability of the antioxidant activity after the juice consumption.

6.7. Bibliography

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CHAPTER VII

CONCLUDING REMARKS

Summary

7.1. Conclusions

Both stinging nettle and winter savory are known medicinal herbs, commonly used in folk medicine as treatment of several ailments. This study aimed to optimize the extraction process, in order to obtain final quality extracts, with high concentration of bioactive compounds and improved biological activities.

- **Optimization study**. The impact of each parameter (pressure level, time of extraction, and solvent (ethanol:water, v:v) concentration) was studied on the concentration of total phenolic compounds, total flavonoids, and on extracts antioxidant activity. Experimental design resorting to response surface methodology showed to be effective on predicting experimental conditions in order to obtain maximum value for each response. Since ethanol concentration was the variable with higher impact in all extractions (followed by pressure level, and extraction time), it was constructed an optimal set of conditions for each solvent concentration, which facilitate the possibility of obtaining different extracts, which one with desirable characteristics. The main results of the optimization study indicate that high pressure assisted extraction (HPE) can be effectively used to increase the concentration of total phenolic compounds, total flavonoids, individual and total chlorophylls, and carotenoids, while it was also observed a clear improvement of antioxidant activity by ABTS, DPPH, and FRAP assays. The gathered data highlighted the superiority of the HPE extraction over the control extractions, and when compared to other studies, it was observed a substantial improvement on the extracts content and their antioxidant activities after HPE. Other advantages can also be spotted, such as the fact that HPE can operate in a considerably short extraction time, since this parameter was the one with lower F-value, thus had fewer impact on the process; for so, a quicker extraction production with improved properties is an indicator that this innovative and environmentally-friendly (produces high quality extracts with no organic solvents in the end, since ethanol is easily recycled) extraction method is a promising alternative for the conventional extraction techniques. These promising results indicate that, by applying the optimal set of conditions predicted for each extract, it is possibly to obtain final extracts with high level of total phenolics, flavonoids, and antioxidant activity, increasing the economic feasibility of the entire process which is highly desirable for the industrial production process.

- **Biological activities**. A clear improvement of all biological activities of the extracts was obtained when the extracts were obtained by high pressure assisted extraction. For stinging nettle, the extracts obtained at 200 MPa, 10 min, 35 and 70% ethanol, were the ones which presented higher concentrations of phenolic acids (5-*O*-caffeoylquinic acid, fertaric and isoferulic acid) and flavonoids (rutin), being these compounds related to the highest antioxidant, DNA-protective, and anti-hypertensive activities. For winter savory, the extracts obtained by high pressure assisted extraction exhibit an interesting range of biological activities, specially the extract obtained at 348 MPa, 20 min, using 35% ethanol. It showed to be the extract with higher concentration of individual phenolic compounds, with higher potential as antioxidant, DNA protective, with ability to inhibit biofilm formation of *E.coli, S. aureus, B. cereus*, and *L. monocytogenes*, and also the only extract to show a value of minimum bactericidal concentration of 20 mg/mL against *L. monocytogenes*. The extracts obtained by HPE also presented a high antioxidant activity, either measured by ORAC assay or by DNA degradation assay, both biological important methods. It is noteworthy that all extracts from both herbs, especially the ones obtained after HPE, were able not only to cause fewer damage in the DNA molecules than the controls, as were also able to protect it against the damage caused by oxidative stress by the presence of reactive oxygen species. Concerning the cytotoxicity of the extracts, it was observed that HPE extracts, in a concentration of 0.5 and 1.0 mg/mL, for winter savory and nettle extracts, respectively, were not considered harmful to HT29 and Caco-2 cell lines (cell culture commonly used to assess the biocompatibility of extracts to human intestinal cells). Nevertheless, in a higher concentration (>1.0 and >5.0 mg/mL for winter savory and nettle extracts, respectively), it was observed that the extracts can significantly reduce the cell viability of a series of cancerous cell lines, indicating that these extracts may present a potential antitumoral activity.

These results scientifically validate the ancient application of both herbs in traditional medicine and demonstrate that emerging technology may be used to obtain more active and safer extracts (either in food or potential cosmetic applications). It can be concluded that all the extracts biological activities are probably caused by phenolic components, such as rosmarinic acid, sagerinic acid, and salvianolic acids A and B.

- **Supplementation of a beverage with an extract.** The supplementation of carrot juice with a winter savory extract allowed to obtain microbiologically safer juices, since they presented lower microbial counts than the non-supplemented ones. Generally, the supplementation of the juice with extract did not affect pH nor the colour parameters. Concerning the total phenolic compounds, total flavonoids, and antioxidant activity (by ABTS, DPPH, and FRAP assays), the values were generally higher in supplemented juices after the 15 days of storage under refrigeration. These data suggest that the addition of winter savory leaves extract (1.0 mg/mL) in carrot juice treated with HPP can effectively improve microbial safety throughout refrigerated storage as well as antioxidant activity, without risking other characteristics of the juice, such as the colour or the acidity.

7.2. Future work

The optimized conditions obtained in this work are important to promote unknown herbs valorization since high-pressure was able to increase antioxidant activity of the extracts and the content of all classes of compounds analysed. Nevertheless, more research should be performed:

- The comparison of HPE results to other emergent extraction technologies, such as pulsed electric fields, ultrasounds, and microwaves should be assessed in order to optimize the process, or to evaluate the possibility to combine technologies in order to obtain even more improved extracts;
- Study the effect of HPE on different biological activities, such as anti-inflammatory activity, which will require *in vivo* studies to validate the positive *in vitro* properties explored in these studies;
- Study the potential of anti-tumoral properties should be explored with more focused analysis, such as the effect of the extracts on NK (natural killer) cells and effect on inflammatory metabolism;
- Since the extracts can be added as supplements for food products, it would be interesting to study their behaviour through the gastrointestinal tract, in order to evaluate if the biological activities are maintained or lost;
- Evaluate the effect of these extracts on skin enzymes, such as elastase and collagenase, and assess their potential as an ingredient of cosmetic products.

APPENDIX A

SUPPLEMENTARY INFORMATION

Table A1. Extensive review on studies present in literature concerning stinging nettle, its composition, and biological activities. This screening allowed to determine the most studied biological activities, as also as the used methodology

	Extraction conditions						Analyses					
Compound	**Extraction method**	**Time (h)**	**Temperature (ºC)**	**Ratio mass to volume (g/mL)**	**Solvent**	**Other extraction methods**	**Antioxidant**	**Anti-microbial**	**Anti-tumoral**	**Other analyses (methods)**	**Results**	**Reference**
Phenolic compounds	-	-	-	1/40	20% Methanol	-	-	-	-	Identification (GC-MS)	-	Kraus and Spiteller (1990)
Urtica dioica agglutinin	-	-	-	-	-	-	-	-	-	Gel filtration; RP-HPLC	Primary sequence of *Urtica dioica* agglutinin	Beintema and Peumans (1992)
Non-starch polysaccharide	-	-	-	-	-	-	-	-	-	-	Obtention of dietary fiber (pectin, hemicellulose, and cellulose)	De Leo et al. (1993)
Urtica dioica agglutinin	-	-	-	-	-	-	-	-	-	NMR	-	Hom et al. (1995)
Bioactive compounds	Solid-liquid extraction	4	50	1/10	Ethanol	-	-	-	Human T-cell line Jurkat; macrophage cell line MonoMac6; epithelial cell line HeLa; mouse L929 fibrosarcosoma cells	-	Inhibition of NF-kB	Riehemann, Behnke and Schulze-Osthoff (1999)
Major compounds	Distillation	6	-	-	Water	-	-	-	-	Separation and identification (GC)	-	Lahigi et al. (2001)
Fatty acids and carotenoids	Adapted for each analysis	-	-	-	Acetyl chloride + Methanol / Acetone + diethyl ether + water	-	-	-	-	Moisture (oven); Identification (HPLC-MS)	Moisture (40.3-82.0%); Total carotenoids (51.4-74.8 ug/g dw); Lutein (60% of total carotenoids)	Guil-Guerrero, Rebolloso-Fuentes and Isasa (2003)
Urtica dioica agglutinin	Sonication	10	-	1/10	5% Acetic acid	-	-	-	-	Identification (HPLC-DAD; LC-MS)	Optimization of HPLC method for lectins analysis	Ganzera, Schönthaler and Stuppner (2003)
Bioactive compounds	-	-	-	-	-	-	-	Mesophilic; Lactic acid (LAB); *Micrococcus* (MC) and *Staphylococcus* (SL); Yeasts and moulds (YM); *Enterobacteriaceae* (ENT)	-	-	Mesophilic inhibition for 3 days; LAB, ENT and YM inactivation; Inactivation of MC and SL after 10 days	Aksu and Kaya (2004)

Table A1. (continued) Extensive review on studies present in literature concerning stinging nettle, its composition, and biological activities. This screening allowed to determine the most studied biological activities, as also as the used methodology

Chlorophylls a and b / β-carotene / lutein	SC-CO_2 (20-28 MPa)	2-12	25-60	0.5-2/-	CO_2 and Ethanol	Chloroform; acetone + Sonication	-	-	-	Identification (HPLC)	Extraction yield (24-73 mg/100 g dw)	Sovová et al. (2004)
Bioactive compounds	Stirring	0.25	~100	1/20	Boiling water	-	FRAP; DPPH; ABTS; Superoxide anion radical; Hydrogen peroxide; Metal chelating activities	Disc diffusion	Antiulcer (ethanol-induced ulcer model)	Phenolic compounds (Folin-Ciocalteu)	Total antioxidant activity higher for nettle extracts than for control (α-tocopherol); Antimicrobial activity against all tested microorganisms	Gülçin et al. (2004)
β -sitosterol and scopoletin	SC-CO_2 (10-28 MPa)	-	25-60	2-4/-	CO_2 and Ethanol	Diethyl ether + Sonication	-	-	-	Identification (HPLC)	Extraction yield (β -sitosterol 0.63 mg/g, scopoletin 0.058 g/g dw)	Sajfrtová et al. (2005)
Chlorophylls a and b	SC-CO_2 (10-30 MPa)	1	25-60	1/10	Petroleum ether; Hexane	Single step extraction	-	-	-	Identification (HPLC)	Extraction yield (14.3-17.3 g/100 g)	Hojnik, Škerget and Knez (2007a)
Chlorophylls a and b	Single step; two step	2	40	1/10	90% Ethanol	Soxhlet; US	-	-	-	Identification (HPLC)	Extraction yield (7.60%)	Hojnik, Škerget and Knez (2007b)
Phenolic compounds	Infusion; Decoction	5 min	~100	1/110	Boiling water	-	ABTS	-	-	Phenolic compounds (Folin-Ciocalteu)	Total phenolic compounds: Powder infusion (0.149 g/L); Leaf infusion (0.163 g/L); Leaf boiling (0.141 g/L)	Giao et al. (2007)
Phenolic compounds; Flavonoids	Maceration	72	RT	1/6.85	70% Ethanol	-	Lipid peroxidation; DPPH	-	-	Phenolic compounds (Folin-Ciocalteu and HPLC)	Total phenolic compounds: 7.62 mg GAE/g dw and Flavonoids: 1.92 mg quercetin/g dw	Hudec et al. (2007)
Phenolic compounds	Solid-liquid extraction	-	RT	-	70% Ethanol (pH 3.2)	-	DPPH	-	-	HPLC-DAD; HPLC-MS; HPLC-MS/MS; Fiber content	Main class in stalks are flavonoids and then anthocyanins (only here)	Pinelli et al. (2008)
Flavonol glycosides; Phenolic acids	Solid-liquid extraction	2	RT	3/20	80% Methanol	-	-	-	-	Flavonol glycosides and phenolic acids Identification (LC-MS); Quantification (RP-HPLC)	High nitrogen levels reduced significantly the concentration of total flavonoids	Grevsen, Frette and Christensen (2008)

Table A1. (continued) Extensive review on studies present in literature concerning stinging nettle, its composition, and biological activities. This screening allowed to determine the most studied biological activities, as also as the used methodology

Bioactive compounds	Solid-liquid extraction	2	20-60	-	Ethanol	-	-	-	-	Chemical characterization (DART TOF-MS); Histamine receptor; Mast cell tryptae; cyclooxygenase; hematopoietic prostaglandin D2 synthase	Proven anti-inflammatory activity against allergic rhinitis	Roschek et al. (2009)
Bioactive compounds	-	-	-	-	-	-	-	-	-	-	Inhibition of NF-kB; Daily feed to rats (30 mg/Kg)	Toldy et al. (2009)
Compound in stinging hairs	-	-	-	-	-	-	-	-	-	Structural changes (SEM)	Rash is caused by both mechanical and biochemical mechanisms	Cummings and Olsen (2011)
Bioactive compounds	Soxhlet (I)	48-72	40-45	1/4	Hexane + chloroform + ethyl acetate + methanol	-	-	Disc Diffusion; MIC; MBC	-	-	Inhibition of various microorganisms (but no fungi); Method I more efficient than Method II	Modarresi-Chahardehi et al. (2012)
	Soxhlet (II)	72	30	1/5	Methanol + chloroform: water + diethyl ether + ethyl acetate + butanol							
Phenolic compounds	Solid-liquid extraction	1	50	1/10	80% Methanol	-	DPPH	-	-	Moisture analysis; Phenolic compounds (Folin-Ciocalteu); Identification (HPLC)	Moisture (81.9/83.1/77.8%); Phenolic compounds (7.62 – 9.91 mg GAE/g dw)	Otles and Yalcin (2012)
Phenolic compounds	Supercritical Ethanol (2 MPa)	2.4	336	1/10	Ethanol	-	-	-	-	Identification (GC-MS); Structural changes (SEM)	Extraction yield (45.3%)	Akalın, Karagöz and Akyüz (2013)
Fatty acids and aminoacids	-	-	-	-	-	-	-	-	-	Moisture; Nitrogen content; Protein; Total fat; Carbohydrates; Calories; Total dietary fiber; Vitamins; Minerals; Aminoacids; Fatty acids	Raw nettle more nutritious than blanched or cooked one	Rutto et al. (2013)
Bioactive compounds	-	-	-	-	Water; Methanol; Dichloro-methane; Hexane	-	-	-	-	Cytotoxicity (MTT); NF- kB Luciferase (in mouse macrophage)	Polar extracts with no anti-inflammatory effect	Johnson et al. (2013)

Table A1. (continued) Extensive review on studies present in literature concerning stinging nettle, its composition, and biological activities. This screening allowed to determine the most studied biological activities, as also as the used methodology

Bioactive compounds	Maceration	1	50	1/10	50% Ethanol	-	DPPH of the extracts and fatty acid composition of the meat	Mesophilic and yeast and moulds on the meat	-	Phenolic compounds (Folin-Ciocalteu)	Used as additive for shelf-life extension of minced meat	Sagir and Turhan (2013)
Phenolic compounds	MW	0.16	-	1/30	Water	Heating with hot plate	DPPH	-	-	Phenolic compounds (Folin-Ciocalteu and HPLC)	24.64 mg GAE/g	Ince, Sahin and Sumnu (2014)
	US	0.5	-	1/30	Water	Maceration		-	-		23.86 mg GAE/g	
Phenolic compounds	Solid-liquid extraction	48	RT	1/8 or 1/15	80% Methanol	-	-	-	-	Identification (HPLC-MS/MS)	5-*O*-caffeoylquinic acid, rutin and isoquercitrin the most abundant	Orcic et al. (2014)
Phenolic compounds	Soxhlet	14-15			96% Ethanol / *n*-hexane	$SC\text{-}CO_2$	DPPH	-	-	Phenolic compounds (Folin-Ciocalteu)	Extraction yields: 14.14% (ethanol) and 0.768% (*n*-hexane)	Koszegi, Vatai and Bekassy-Molnar (2015)
Phenolic compounds	Maceration	0.63	25	1.25/25	54% methanol	US	DPPH	-	-	Phenolic compounds (Folin-Ciocalteu); Identification and quantification (HPLC; LC-MS)	9.9 mg GAE/g dw	Vajić et al. (2015)
Protein fraction (UDHL30)	-	-	-	-	-	-	ABTS; superoxide anion	-	Ames test	Cytotoxicity (MTT)	No cytotoxic nor mutagenic effects; great scavenger activity	Di Sotto et al. (2015)
Phenolic compounds	Infusion; Maceration; Decoction	0.5; 24; 0.4	80; RT; 100	1/100	Distilled water	-	FRAP; ABTS	-	Human colon-cancer cells (SW480)	Carbohydrates; Minerals; Phenolic compounds (Folin-Ciocalteu + HPLC)	Higher yield after infusion; with cytotoxic and antioxidative properties against cancer cells	Belscak-Cvitanovic et al. (2015)
Phenolic compounds	Infusion	-	-	1/100	Boiling water	Solid-liquid extraction (Ethanol)	FRAP	-	-	Phenolic compounds (Folin-Ciocalteu)	Aqueous extracts with higher concentration of phenolics than ethanolic extracts	Koczka, Petersz and Stefanovits-Bányai (2015)
Bioactive compounds	Soxhlet	6	77	1/20	50% Methanol	Maceration and Soxhlet	DPPH; FRAP; Hydrogen peroxide	-	-	Phenolic compounds (Folin-Ciocalteu); Flavonoids (colorimetry)	Higher phenolics concentration after UAE and higher flavonoids concentration after Heat reflux	Stanojević et al. (2016)
	Heat reflux	2	25	1/20	30, 50, 80, 100% Methanol							
	UAE	1	25	1/20	50% Methanol							

Table A1. (continued) Extensive review on studies present in literature concerning stinging nettle, its composition, and biological activities. This screening allowed to determine the most studied biological activities, as also as the used methodology

Biomineral formations	-	-	-	-	-	-	-	-	-	Structural changes (SEM)	Phytolites (silicon) located at the bases of stings	Golokhvast (2016)
Bioactive compounds	Soxhlet	72 h	60-80	-	Petroleum benzene + chloroform + methanol	-	-	-	-	Colour; pH; Appearance; Texture; Rheology; *in vivo* anti-inflammatory and analgesic studies	Root extract showed analgesic and anti-inflammatory activities in mice	Liao et al. (2016)
Phenolic compounds	Solid-liquid extraction	0.5-2	25-65	1/40	50-100% Ethanol	-	FRAP; Cupric reducing antioxidant capacity (CUPRAC)	-		Phenolic compounds (Folin-Ciocalteu); Anti-aging (collagenase and elastase)	Antiaging effect by inhibition of enzyme activities (elastase and collagenase) due to the presence of ursolic acid and quercetin	Bourgeois et al. (2016)
Chemical profile (Chlorophylls, total carotenoids, fatty acids, elements (metal and non-metal))	Hydro-distillation	2	-	40g	Distilled water (and petrol ether to trap volatile compounds)	-	-	-	-	Chemical analysis (GC-MS-FID)	12 monoterpenes detected (3 quantified)	Đurović et al. (2017)
	Soxhlet	-	-	1/30	96% Ethanol	-	-	-	-	Chlorophylls and carotenoids	Extraction yield (21.75%)	
	UAE	1	45	1/30	96% Ethanol	-	-	-	-	Fatty acids (GC-FID); elemental analysis (mercury analyser)	Extraction yield (3.65%)	
Phenolic compounds	Maceration	12	RT	1/10	Ethanol	-	-	-	-	Phenolic compounds (phosphomolibdate assay; HPLC)	120.92 mg GAE/100 g FW	Dobrinas, Stanciu and Lupsor (2017)
Phenolic compounds	Solid-liquid extraction (with agitation)	1	20	1/10	Methanol: water:HCl (79:20:1, v/v/v/)	-	-	-	-	Phenolic compounds (Folin-Ciocalteu)	Fresh nettle (743.4 mg GAE/100g FW)	Augspole et al. (2017)

Table A1. (continued) Extensive review on studies present in literature concerning stinging nettle, its composition, and biological activities. This screening allowed to determine the most studied biological activities, as also as the used methodology

Bioactive compounds	UAE	0.75	-	1/30	Water	-	DPPH; reducing power; direct current polarographic assay	MIC; antifungal activity	-	Phenolic compounds (Folin-Ciocalteu and UHPLC-DAD-HESI-MS/MS); Flavonoids (colorimetry); Cytotoxicity (MTT assay)	Phenolics (463.6 mg CAE/g); Flavonoids (11.00 mg CE/g); DPPH (16.93 ug/mL)	Zekovic et al. (2017a)
	MAE	0.75	-	1/30	Water	-						
	Subcritical H_2O	0.75	-	1/30	Water	-						
Bioactive compounds	Solid-liquid extraction (in water bath)	1	70	-	70% Ethanol	-	-	Disc diffusion; MIC	-	Phenolic acids, flavonoids, flavones and flavonols (HPLC-DAD/Vis); Phenolic compounds (Folin-Ciocalteu); Flavonoids (colorimetry)	Major compound (Caffeic acid, 163.01 ± 3.63 µg/g); Phenolics (25.85 ± 1.2 mg GAE/g); Flavonoids (22.47 ± 0.7 mg CAE/g)	Zenão et al. (2017)
Fibre yarns	-	-	-	-	-	-	-	-	-	Tensile strength; Elongation; Friction strength; Structural changes (SEM)	The highest tensile strength, elongation and friction strength properties = microwave energy method	Sansal et al. (2017)
Bioactive compounds	Stirring at RT	48	-	1/10	95% Ethanol	-	Lipid peroxidation (TBARS)	Shelf-life evaluation	-	Phenolic compounds (Folin-Ciocalteu); Texture; Colour; sensorial analysis	Sausage incorporated with nettle extract had the highest sensory score regarding flavour, freshness odour and overall acceptability	Alirezalu et al. (2017)
Bioactive compounds (review article)	-	-	-	-	-	-	Mainly due to the presence of quercetin, rutin and ascorbic acid	Against Gram-positive bacteria, gram–negative bacteria and some yeast	Antiulcer (ethanol-induced ulcer model)	-	Anti-inflammatory effect due to its inhibitory effect on NF-κB activation; may be due to the presence of quercetin	Jan, Zarafshan and Singh (2017)
Bioactive compounds	Boiling; sonication	0.5; 0.25	~100	1/20	Hot water and ethanol	-	FRAP; DPPH	-	-	a-amylase, a-glucosidase and formation of advanced glycation end products (AGE) inhibition assays in vitro; Phenolic compounds (Folin-Ciocalteu and UPLC-MS/MS)	Total phenolic compounds (27.7 mg GAE/L)	Sekhon-Loodu and Rupasinghe (2019)

Table A2. Extensive review on studies present in literature concerning winter savory, its composition, and biological activities. This screening allowed to determine the most studied biological activities, as also as the used methodology

	Extraction conditions						Analyses					
Compound	**Extraction method**	**Time (h)**	**Temperature (ºC)**	**Ratio mass to volume (g/mL)**	**Solvent**	**Other extraction methods**	**Antioxidant**	**Anti-microbial**	**Anti-tumoral**	**Other analyses (methods)**	**Results**	**Reference**
Water-soluble substances	Solid-liquid extraction	24	RT	1/60	Acetone Ethanol + 70% Ethanol:water (successively)	-	-	-	-	-	Strong anti-HIV-1 activity with a dose of 16 µg/mL extract	Yamasaki et al. (1998)
Essential oil	Hydro-distillation	0.83	-	-	-	-	-	MIC	-	Identification (GC)	Carvacrol identified as the major compound by GC; low inhibitory concentrations	Ciani et al. (2000)
Volatile compounds	Hydro-distillation	3	-	100/-	-	-	β-carotene bleaching; thiobarbituric acid (TBA)	-	-	Identification (GC-MS)	21 compounds identified (97.4% of the total oil); Major compound was monoterpene thymol (45.2%)	Radonic and Milos (2003)
Essential oil	Hydro-distillation	3	-	100/-	-	-	-	MIC; MBC	-	Identification (GC-MS)	32 compounds identified; Major compound was the phenolic monoterpene, carvacrol (45.7%).	Skocibusic and Bezic (2004)
-	Solid-liquid extraction	48	RT	1/100	70% Methanol	-	DPPH	-	Sulforhodamine B assay (cell lines HeLa, MCF-7, HT29)	-	Antiproliferative effect on HeLa cell line with IC_{50} ranging from 0.41 to 0.84 mg/mL	Cetojevic-Simin et al. (2004)
Essential oil	Hydro-distillation	-	-	-	-	-	-	MIC	-	Identification (GC-MS)	MIC of 500 ppm after 7 d; β-phellandrene was the most interesting component with a MIC of 50 ppm. The most active of phenols was carvacrol (MIC of 100 ppm)	Tampieri et al. (2005)
Bioactive compounds	Maceration	2x24	RT	1/25	70% Methanol; consecutive extractions with Petroleum ether, Chloroform, Ethyl acetate, and *n*-Butanol	-	Hydroxyl radicals by Fenton reaction	Disc diffusion and microbroth dilution	-	-	*n*-Butanol extract had the best antioxidant activity (100% at 0.5 mg/mL in Fenton reaction system	Ćetković et al. (2007a)

Table A2. (continued) Extensive review on studies present in literature concerning winter savory, its composition, and biological activities. This screening allowed to determine the most studied biological activities, as also as the used methodology

Phenolic compounds	Maceration	2x24	RT	10/2x20	70% Methanol; consecutive extractions with Petroleum ether, Chloroform, Ethyl acetate, and *n*-Butanol	-	-	-	-	Phenolic compounds (Folin-Ciocalteu and HPLC)	Higher concentration of total phenolic in ethyl acetate (47.59 mg/g) and *n*-butanol (96.70 mg/g)	Ćetković et al. (2007b)
Essential oil	Hydro-distillation	4	-	90/-	-	-	-	-	-	Identification and quantification (GC-MS)	The largest amount of the essential oil (3.0%) was extracted from summer savory (*S. hortensis*); the lowest amount (1.4%) was obtained from winter savory (*S. montana*)	Omidbaigi, Rahimi and Kazemi (2007)
Essential oil	Steam distillation	3	-	-	-	-	-	Agar diffusion; micro-atmosphere	-	Identification and quantification (GC and GC-MS)	35 compounds were identified by GC-MS, and carvacrol (18.0%), *p*-cymene (14.3%), and thymol (9.9%), were the most abundant ones	Fraternale et al. (2007)
Essential oil	Microwave (220-660 W) assisted hydro-distillation (MAHD)	-	-	1/20	Distilled water	Hydro-distillation	-	-	-	-	The extraction yield obtained by MAHD was similar to that obtained by hydrodistillation (0.7%); Extraction more efficient at higher power levels	Rezvanpanah et al. (2008)
Volatile fraction	SC-CO_2 (90 bar)	-	40	100/-	CO_2 (1.1 kg/h)	Hydro-distillation	-	MIC; MLC	-	Identification (GC-MS)	Growth inactivation of *Bacillus subtilis* and *Bacillus cereus*, showing some activity against *Botrytis* spp. and *Pyricularia oryzae*	Silva et al. (2009)
Non-volatile fraction	SC-CO_2 (250 bar)	4	´-	5/-	CO_2	Soxhlet	-	-	-	Alzheimer's disease (Ellman's assay); Identification and quantification (HPLC-DAD)	High content of catechin, chlorogenic, vanillic, and protocatechuic acids; Selective inhibition of butyrylcholinesterase	

Table A2. (continued) Extensive review on studies present in literature concerning winter savory, its composition, and biological activities. This screening allowed to determine the most studied biological activities, as also as the used methodology

Volatile fraction	SC-CO_2 (90-100 bar)	-	40-50	-	CO_2 (0.8-1.3 kg/h)	Hydro-distillation	-	-	-	Structural changes (SEM); Identification and quantification (GC / GC-MS)	Higher concentrations of carvacrol, thymol, and *p*-cymene for hydrodistillation	Grosso et al. (2009b)
Essential oil	Infusion	0.16; 0.20; 0.25	-	1/110	Boiling water	-	ABTS	-	-	Identification and quantification (LC-MSMS)	Maximum rate of extraction ranged from 0.3 to 1.6 g/L, and increased when the particle size decreased	Gião et al. (2009)
Volatile fraction	SC-CO_2 (90 bar)	-	40	100/-	CO_2 (1.1 kg/h)	Hydro-distillation	-	-	-	Herbicidal assay; Identification (GC-MS)	Promising results as new natural herbicide for uncultivated fields	Grosso et al. (2010)
Non-volatile fraction	Maceration	72	20	1.5/10	Ethanol and water	Soxhlet	DPPH; FRAP	Disc diffusion; MIC	-	Phenolic compounds (Folin-Ciocalteu); Identification and quantification (GC-MS)	Major volatile constituents of the essential oil were carvacrol (306 g/L), and thymol (141 g/L); Higher antioxidant activity for hot water extracts	Serrano et al. (2011)
Volatile fraction	Hydro-distillation	3	-	1/7	Deionised water							
Volatile fraction	SC-CO_2 (90-120 bar)	4	40 and 50	40-100/-	CO_2	Soxhlet; Hydro-distillation	-	-	-	Alzheimer's disease; Volatile fraction (GC-MS); Non-volatile fraction (HPLC-DAD)	Higher concentrations of carvacrol, thymol, and *p*-cymene for hydrodistillation	Palavra et al. (2011)
Essential oil	Hydro-distillation	2	-	-	-	-	DPPH	Disc diffusion; MIC	-	Identification (GC-MS and 1H NMR spectra)	43 compounds identified; Major components were carvacrol (44.5%), *p*-cymene (16.9%), and *g*-terpinene (8.7%)	Marin et al. (2012)
Volatile fraction	SC-CO_2 (8-10 MPa)	-	40-50	70-120/-	CO_2 (0.71 -1.64 kg/h)	Hydro-distillation	-	-	-	Identification (GC; GC-MS)	The major differences between both extracts is the presence of cuticular waxes and the relative amount of thymoquinone	Coelho et al. (2012)

Table A2. (continued) Extensive review on studies present in literature concerning winter savory, its composition, and biological activities. This screening allowed to determine the most studied biological activities, as also as the used methodology

Quercetin and rosmarinic acid	Uniform stirring	-	-	1/100	Boiling distilled water	-	-	-	-	Identification (HPLC)	Precise method at the intra-day and inter-day levels, (recovery rate 90.5%)	da Silva et al. (2013)
Bioactive compounds	Ultrasonic bath	0.5	-	1/10	Ethanol	-	DPPH; total antioxidant capacity	-	-	Alzheimer's disease (Ellman's assay); Identification and quantification (RP-HPLC)	Rosmarinic acid was the predominant constituent; High antioxidant activity; 75% inhibition at 1 mg/mL on Ellman's colorimetric assay	Vladimir-Knežević et al. (2014)
Essential oil	Hydro-distillation	3	-	-	Water	-	DPPH; ORAC; ABTS; FRAP	MIC; MBC; MFC	-	Clinical trial (modified Ellman's method – human serum cholinesterase); Identification (GC-MS)	With increase of the growth altitude, the content of phenolics (carvacrol and thymol) are either decreased or their ratio is changed	Mihajilov-Krstev et al. (2014)
Essential oil	Hydro-distillation	-	-	-	-	-	DPPH	-	-	Identification (GC-MS)	Phytochemical profiles indicated that the *S. montana* essential oil belong to the carvacrol chemotype	Jianu et al. (2015)
Rosmarinic acid	Maceration	0.083	-	1/100	Boiling water	-	ABTS; ORAC	-	-	Structural changes (SEM / DSC) Identification (HPLC)	Individual and small sizing chitosan nanoparticles were obtained	da Silva et al. (2015)
Essential oil	Hydro-distillation	3	-	1/15	Bi-distilled water	-	DPPH; ABTS; reducing power	-	-	Identification (GC and GC-MS)	Carvacrol (63.40%) was the most abundant compounds, followed by *para*-cymene (10.97%) and γ-terpinene (3.70%)	Trifan et al. (2015)
Phenolic compounds	Solid-liquid extraction	-	-	1/10	Methanol; ethanol; acetone	-	DPPH; ABTS	-	-	Phenolic compounds (Folin-Ciocalteu and HPLC–DAD–ESI–TOF–MS)	45 compounds were identified, 42 of which were identified for the first time; Chlorogenic acid was the most abundant compound	López-Cobo et al. (2015)

Table A2. (continued) Extensive review on studies present in literature concerning winter savory, its composition, and biological activities. This screening allowed to determine the most studied biological activities, as also as the used methodology

Phenolic compounds	Soxhlet	-	-	200/-	Chloroform and methanol	-	DPPH; ABTS; FRAP; Molybdenum ions; Metal-chelating ability	-	-	Phenolic compounds (Folin-Ciocalteu); Flavonoids (colorimetry); Particular phenolic acids (HPLC–UV)	Source of natural phenolic compounds, with significant antioxidant activities	Zeljković et al. (2015)
Phenolic compounds	Shaking water bath	1.5	75	0.15/25	50% Methanol	-	DPPH; FRAP	-	-	Phenolic compounds (Folin-Ciocalteu); Flavonoids (colorimetry)	Total phenolics (68.1 to 102.6 mg/g dw); Total flavonoids (38.3 to 67.0 mg/g dw); 61 compounds identified: main: myrcene, *p*-cymene, thymol, and carvacrol	Hajdari et al. (2016)
Essential Oil	Hydro-distillation	3	-	1/10	-	-	-	-	-	Identification (GC-FID and GC-MS)		
-	Infusion and stirring	0.17	85	60/-	Water	-	-	-	-	-	Clinical trials in rats	Masuda et al. (2016)
Bioactive compounds	SC-CO_2 (100-350 bar)	4.5	40-60	60/-	CO_2 (0.194 kg/h)	Soxhlet	-	-	-	Extraction yield; Identification (GC-MS)	Carvacrol as the most concentrated compound	Vladić et al. (2016)
Essential oil	Hydro-distillation	2	-	80/-	-	-	-	MIC; MBC	-	Identification and quantification (GC-FID)	Highest essential oil yield after SC-CO_2 extraction as well as the highest content of thymol and carvacrol. Antimicrobial activity was the same or weaker when comparing both methods	Damjanovic-Vratnica et al. (2016)
Bioactive compounds	SC-CO_2 (100 bar)	6	40	80/-	CO_2 (0.3 kg/h)							
Rosmarinic acid	Solid-liquid extraction	-	-	-	Water	-	-	-	-	-	Chitosan nanoparticles used to encapsulate antioxidant rosmarinic acid	da Silva et al. (2016)
Essential oil	SC-CO_2 (100-300 bar)	4	40	50/-	CO_2 (0.2 kg/h)	-	DPPH	-	Human cancer cell lines (HeLa, MDA-MB-453, K562) and normal cell lines MRC-5 (MTT assay)	Moisture; Identification (GC-MS and GC-FID)	Carvacrol as the most concentrated compound	Elgndi et al. (2017)

Table A2. (continued) Extensive review on studies present in literature concerning winter savory, its composition, and biological activities. This screening allowed to determine the most studied biological activities, as also as the used methodology

Bioactive compounds	Subcritical water extraction	0.35	200	1/10	Water	-	DPPH	-	-	Phenolic compounds (Folin-Ciocalteu); Flavonoids (colorimetry); Volatile fraction (GC-MS)	Total phenols, total flavonoids, and IC_{50} obtained were found to be 11.24 g/100 g, 6.84 g/100 g and 0.0028 mg/mL, respectively.	Vladić et al. (2017)
Phenolic compounds	Microwave	0.016-0.42	-	1/10	70% Ethanol	-	DPPH; reducing power	-	-	Phenolic compounds (Folin-Ciocalteu); Flavonoids (colorimetry)	Microwaves proved to be suitable for fast and effective extraction of total phenolics	Zekovic et al. (2017a)
Essential oil	Hydro-distillation	-	-	-	-	-	-	MIC; MBC	-	GC-MS	Antimicrobial activity is attributed to the presence of carvacrol	Babaei et al. (2018)

Table A3. Extensive review on studies present in literature concerning high pressure assisted extraction (HPE). From this screening it was possible to choose the most important independent variables (factors) and respective levels used to perform the response surface methodology

			Extraction conditions (HPE)						Analyses					
Material	**Extracted compounds**	**Pre-treatment**	**Pressure (MPa)**	**Time (min)**	**Temperature (ºC)**	**Ratio Mass to volume (g/mL)**	**Solvent**	**Other extraction methods**	**Antioxidant**	**Anti-tumoral**	**Anti-microbial**	**Structural changes**	**Other analyses (methods)**	**Reference**
Tomato puree	Carotenoids (lycopene, β-carotene, total carotenoids)	Crude	400	15	25	-	Water	-	DPPH; Lipid oxidation	-	-	-	pH; Titratable acidity; Soluble solids; Total solids; Colour; HPLC	Shouqin, Junjie and Changzhen (2004)
Propolis	Flavonoids	Crude	500	1	RT	1/35	75% Ethanol	RT; Heat reflux	-	-	-	-	Total flavonoids (colorimetry)	Sánchez-Moreno et al. (2004)
American Ginseng	Ginsenosides	Dried in vacuum	200	2	25	1/50	60% Ethanol	US; MW; SC-CO_2; Soxhlet; Heat reflux	-	-	-	-	HPLC	Shouquin et al. (2006)
Tomato paste	Lycopene	Dried in air drier	500	1	RT	1/5	50% Ethanol	Solid-liquid extraction with sonication	-	-	-	-	HPLC	Xi (2006a)
Tomato paste	Lycopene	Dried in air drier	500	1	RT	1/6	75% Ethanol	Solid-liquid extraction with sonication	-	-	-	-	HPLC	Xi (2006b)
Propolis	Flavonoids	Crude	500	1	RT	1/35	75% Ethanol	Leaching at RT; Heat reflux	β-carotene bleaching; DPPH	-	-	-	Phenolic compounds (Folin-Ciocalteu)	Xi (2006c)
Ginseng	Ginsenosides	Dried	500	2	RT	1/75	50% Ethanol	RT; US; SC-CO_2	-	-	-	-	-	Shouqin, Ruizhan and Changzheng (2007)
Rhodiola sachalinensis	Flavonoids; salidroside	Dried in vacuum	500	3	RT	1/70	60% Ethanol	US; Leaching; Soxhlet; Heat reflux	DPPH	-	-	-	HPLC	Zhang, Bi and Liu (2007)
Propolis	Phenolic compounds; Flavonoids	Crude	500	1	RT	1/35	75% Ethanol	Leaching at RT; Heat reflux	β-carotene bleaching; DPPH	-	-	-	Phenolic compounds (Folin-Ciocalteu); Total flavonoids (colorimetry)	Xi and Shouqin (2007)
Sour cherry pomace	Phenolic compounds	Crashed, heated and pressed	176-193	25	60	1/15	Ethanol	SC-CO_2; Solid-liquid extraction	DPPH	-	-	-	Phenolic compounds (Folin-Ciocalteu)	Adil, Yener and Bayındırlı (2008)
Grape waste	Anthocyanins	Whole skins	600	60	70	1/4.5	50% Ethanol	PEF; US	ABTS	-	-	-	LC-DAD/ESI-MS; Phenolic compounds (Folin-Ciocalteu)	Corrales et al. (2008)
Grape waste	Anthocyanins	Whole skins	600	30	70	1/4.5	50% Ethanol	-	ABTS	-	-	-	HPLC-DAD/ESI-MS	Corrales et al. (2009)

Table A3. **(continued)** Extensive review on studies present in literature concerning high pressure assisted extraction (HPE). From this screening it was possible to choose the most important independent variables (factors) and respective levels used to perform the response surface methodology

Ginseng	Ginsenosides	Dried in oven	200	5	60	1/50	70% Ethanol	MW; US; Soxhlet; Heat reflux	DPPH	-	-	Scanning electron microscope (SEM)	Colorimetry	Chen et al. (2009)
Rhodiola sachalinensis	Salidroside	Dried in vacuum	300	3	25	1/50	60% Ethanol	Cellulase; US; Leaching; Heat reflux; Soxhlet	-	-	-	-	-	Bi et al. (2009)
Green tea	Phenolic compounds	Dried in vacuum	500	1	RT	1/20	50% Ethanol	RT; US; Heat reflux	-	-	-	-	Phenolic compounds (Folin-Ciocalteu)	Xi et al. (2009)
Green tea	Caffeine	Dried in vacuum	500	1	RT	1/20	50% Ethanol	RT; US; Heat reflux	-	-	-	-	-	Xi (2009)
Litchi fruit pericarp	Phenolic compounds	Dried in hot air oven	500	2.5	70	1/50	85% Ethanol	US; RT	DPPH; Superoxide anion	-	-	-	Phenolic compounds (Folin-Ciocalteu)	Prasad et al. (2009e)
Longan fruit pericarp	Phenolic compounds	Dried in hot air oven	500	2.5	50	1/50	50% Ethanol	RT	DPPH; Superoxide anion	-	-	-	Gallic acid calibration	Prasad et al. (2009d)
Longan fruit pericarp	Phenolic compounds	Dried in hot air oven	500	2.5	30	1/50	50% Ethanol	RT	Phosphomolybdenum; lipid peroxidation; DPPH; superoxide anion	MTT assay Cell lines (HepG2, A-549, SGC-7901)	-	-	Gallic acid calibration; Identification (HPLC)	Prasad et al. (2009a)
Longan fruit pericarp	Corilagin	Dried in hot air oven	500	2.5	30	1/50	50% Ethanol	US; Solid-liquid extraction	-	-	-	-	Identification (HPLC)	Prasad et al. (2009c)
Litchi fruit pericarp	Flavonoids	Dried in hot air oven	400	30	25	1/40	Ethanol: HCl (85:15)	US; Solid-liquid extraction	DPPH; Superoxide anion	-	-	-	Phenolic compounds (Folin-Ciocalteu); Identification (HPLC)	Prasad et al. (2009b)
Longan fruit pericarp	Polysaccharides; Lignins; Cellulose	Dried in freeze dryer	500	30	25	1/15	Distilled water	Control (0.1 MPa, 25ºC, 30 min)	-	-	-	-	Isolation of polysaccharides and cellulose; Acid hydrolysis of cellulose	Yang et al. (2009)
Schisandra chinensis	Deoxy-schisandrin; *y*-schisandrin	Dried in vacuum	400	5	RT	1/90	90% Ethanol	Heat reflux; US	DPPH	-	-	-	Identification (HPLC)	Liu, Zhang and Wu (2009)

Table A3. **(continued)** Extensive review on studies present in literature concerning high pressure assisted extraction (HPE). From this screening it was possible to choose the most important independent variables (factors) and respective levels used to perform the response surface methodology

Berberis koreana	Phenolic compounds	Crude	500		RT	1/10	Water	US; Solid-liquid extraction	DPPH; Xanthine oxidase	Cell lines (A549, MCF-7, Hep3B, AGS, HEK293); Human NK cell growth; Nitric oxide production	-	-	HPLC; Phenolic compounds (Folin-Ciocalteu)	Qadir et al. (2009)
Korean barberry	Phenolic compounds	Dried stem brought	500	30	30	1/90	Distilled water	Solid-liquid extraction	-	Ames Salmonella mutagenicity	Probiotic activity; MIC	-	pH; Phenolic compounds (Folin-Ciocalteu)	Lee, He and Ahn (2010)
Deodeok roots	Phenolic compounds and Flavonoids	Dried in cabinet-type convective drier, and grinded	500	30	50	-	70% Ethanol	Solid-liquid extraction	DPPH; Ferric reducing power	Ames Salmonella mutagenicity	Probiotic activity; MIC and MBC	-	pH; Phenolic compounds (Folin-Ciocalteu); Identification (HPLC)	He et al. (2010)
Longan fruit pericarp	Phenolic compounds	Dried in hot-air oven	500	30	30	1/50	50% Ethanol	US; Solid-liquid extraction	DPPH; Reducing power; Total antioxidant activity; Superoxide anion radical; Lipid peroxidation	-	-	-	Identification (HPLC)	Prasad et al. (2010b)
Bitter melon	Momordicosides	Dried in hot air oven	423.1	7	30	1/45.3	70% Ethanol	Heat reflux	-	-	-	-	Total momordicosides (UV/Vis); HPLC	Ji et al. (2010)
Ginseng	Ginsenosides	Dry powder (purchased)	600	5	RT	-	Water	RT	-	-	-	-	Identification (HPLC)	Shin et al. (2010)
Longan fruit pericarp	Phenolic compounds	Dried in hot air oven	500	30	30	1/50	50% Ethanol	RT	DPPH; Superoxide anion; Phospho-molybdenum	-	-	-	Identification (HPLC)	Prasad et al. (2010a)
Green tea	Catechins and caffeine	Dried in vacuum	400	15	RT	1/20	50% Ethanol	Solid-liquid extraction	-	-	-	-	HPLC	Xi et al. (2010)

Table A3. **(continued)** Extensive review on studies present in literature concerning high pressure assisted extraction (HPE). From this screening it was possible to choose the most important independent variables (factors) and respective levels used to perform the response surface methodology

Ginseng	Ginsenosides	Fresh roots *versus* Dried roots	80	12 h	30	1/20	Water	Heat extraction	-	-	-	-	Ginsenosides (HPLC); Phenolic compounds (Folin-Ciocalteu); Total sugars (phenol-H_2SO_4); Volatile compounds (GC-MS)	Lee et al. (2011)
Deodeok	Phenolic compounds; Flavonoids	Inlet air temperature	300	20	30	1/5	Water	Heat extraction	DPPH	Sulforhod-amineB assay Cell line [HEK-293]	-	-	Phenolic compounds (Folin-Ciocalteu); Identification (HPLC)	He et al. (2011)
Green tea	Phenolic compounds	Fresh leaves pulverized	400	15	RT	1/20	50% Ethanol	-	-	-	-	SEM; TEM	-	Xi et al. (2011a)
Epimedium koreanum Nakai	Flavonoids	Dry powder (purchased)	350	5	-	-	50% Ethanol	Ultrasounds, Heat reflux, SC-CO_2	-	-	-	-	Total flavonoids (colorimetry)	Hou et al. (2011)
Green tea	Phenolic compounds	Dried in hot air oven	450	5	RT	1/20	50% Ethanol	Solid-liquid extraction	DPPH; Phospho-molybdenum	-	-	-	Phenolic compounds (Folin-Ciocalteu)	Xi et al. (2011b)
Cedrus male cones	Pollen protein	Air dried	330	30	RT	1/5	0.2M PBS	-	-	-	-	Light microscopy; SEM	Bradford	Altuner, Çeter and Alpas (2012)
Orange peel	Pectin	Vacuum drying oven	500	10	55	1/50	Water	Heat extraction; MW	-	-	-	Rheology (viscosity)	Gelling properties; Activation energy; Degree of esterification	Guo et al. (2012)
Dendrobium candidum	Polysaccharides	Fresh flowers	445.3	6.7	-	1/237.9	-	Heat reflux	-	-	-	-	-	Tao et al. (2012)
Dysosma versipellis	Podophyllotoxin; 4'-demethyl-podophyllotoxin	Crude	200	1	-	1/12	80% Methanol	Heat reflux	-	-	-	-	HPLC; ESI-MS; NMR	Zhu et al. (2012)
Green tea	Phenolic compounds	Dried in hot air oven	500	15	RT	1/20	50% Ethanol	-	-	-	-	-	Phenolic compounds (Folin-Ciocalteu)	Xi et al. (2013)
Beer wort	Xanthohumol	Boiled	250	5	25	-	-	Boiling	-	-	-	-	HPLC-UV/Vis	Santos et al. (2013)

Table A3. **(continued)** Extensive review on studies present in literature concerning high pressure assisted extraction (HPE). From this screening it was possible to choose the most important independent variables (factors) and respective levels used to perform the response surface methodology

Honey pomelo	Pectin	Dried in a vacuum freeze dyer	500	10	55	1/50	Distilled water + 0.5M hydro-chloric acid	High-speed shearing; Thermal extraction	-	-	-	Viscosity; Light microscopy; Emulsion stability	Galacturonic acid; Degree of esterification; Protein content; Molecular weight	Guo et al. (2014)
Mango peel	Mangiferin; Lupeol	Freeze dried	150	20	25	1/10	80% Ethanol; Hexane	Maceration; Soxhlet; US; MW	-	-	-	-	HPLC	Ruiz-Montanez et al. (2014)
Lemon peels	Phenolic compounds	Crude	500	3	10	-	-	Control (0.1 MPa, 3-10 min)	DPPH	-	-	-	Phenolic compounds (Folin-Ciocalteu)	Casquete et al. (2014)
Citrus peels	Phenolic compounds	Crude	300	3	10	-	-	Control (0.1 MPa, 3-10 min)	DPPH; ABTS	-	Growth inhibition (Halo formation)	-	Phenolic compounds (Folin-Ciocalteu)	Casquete et al. (2015)
Tomato waste	Total carotenoids; Lycopene	Air dried and crushed	700	10	25	1/4	Ethyl lactate	Control (0.1 MPa, 25ºC, 30 min)	-	-	-	-	Total carotenoids (colorimetry); Lycopene (HPLC)	Strati, Gogou and Oreopoulou (2015)
Chilean papaya seeds	Antioxidants; Sulforaphane; Fatty acids	Air died in dark	500	15 (pulses of 1 min)	RT	-	80% Methanol	Solid-liquid extraction; US	DPPH; FRAP	-	-	-	Moisture; Protein content; Lipid content; Fiber; Ash; Phenolic compounds; Total flavonoids; Sulforaphane; Oil extraction	Briones-Labarca et al. (2015)
Orange peel	Phenolic compounds; Flavonoids	Freeze dried	50	30	35	1/10	80% Ethanol	Solid-liquid extraction; US; MW; SC-CO_2	ABTS	-	-	-	Phenolic compounds (Folin-Ciocalteu); Total flavonoids (colorimetry); Identification (HPLC)	M'hiri et al. (2015)
Moringa seeds	Essential oil	Purchased and cleaned	19.63	27.17	85.57	-	Water	-	-	-	-	-	Moisture content and yield	Fakayode and Ajav (2016)

Table A3. **(continued)** Extensive review on studies present in literature concerning high pressure assisted extraction (HPE). From this screening it was possible to choose the most important independent variables (factors) and respective levels used to perform the response surface methodology

Shrimp waste	Astaxanthin	Shells separated from flesh and vacuum dried	200	5	-	1/20	Ethanol	Solid-liquid extraction	DPPH; superoxide anion radical	-	-	SEM	-	Li et al. (2016)
Passion fruit peel	Pectin	Grounded and dried in oven with air circulation	300	20	50	1/30	Nitric acid, pH 1.0	Pressure pre-treatment + high temperature / Heat extraction	-	-	-	-	Pectin purification; Galacturonic acid; Degree of esterification; Apparent viscosity	Oliveira et al. (2016)
Egg yolk	5-methyl-tetrahydrofolate	Fresh white shelled eggs	400	5	RT	1% solids	Mili-Q water	-	-	-	-	-	Total nitrogen content; RP-HPLC; Electrophoresis	Naderi et al. (2017)
Garden pansy	Phenolic compounds	Freeze-dried and grounded	384	15	RT	1/30	35% Ethanol	-	Total reducing capacity (Folin-Ciocalteau); DPPH	-	-	-	Flavonoids (colorimetry); Hydrolysable tannins (colorimetry); Total monomeric anthocyanins (pH differential)	Fernandes et al. (2017)
Pomegranate peel	Phenolic compounds	Dried in a laboratory incubator with air circulation	356-600	30	RT	1/15	32-56% Ethanol	-	DPPH; ABTS; FRAP	-	-	-	Phenolic compounds (Folin-Ciocalteau); Total condensed tannins (vanillin method); Total flavonoids (Dowd method); Total anthocyanin (pH differential); Identification (uHPLC and LC-DAD/ESI-MS)	Alexandre et al. (2017b)
Fermented fig	Phenolic compounds	Dried and grounded	600	5-30	RT	1/15	<15% Ethanol	-	DPPH; ABTS; FRAP	-	-	-	Phenolic compounds (Folin-Ciocalteau); Total condensed tannins (Vanillin method); Total flavonoids (Dowd method); Identification (LC-DAD/ESI-MS, and HPLC-DAD)	Alexandre et al. (2017c)

Table A3. **(continued)** Extensive review on studies present in literature concerning high pressure assisted extraction (HPE). From this screening it was possible to choose the most important independent variables (factors) and respective levels used to perform the response surface methodology

Japanese raisin tree	Total phenolic acids	-	400	60	-	-	-	Hot water extraction	-	-	-	-	Enzymes: alcohol dehydrogenase, aldehyde dehydrogenase, glutathione-S-transferase	Lee (2017)
Blue honeysuckle berries	Anthocyanins	-	426	7	-	1/14.7	-	Ultrasound assisted extraction; Solid-liquid extraction	DPPH; ABTS; FRAP	-	-	-	Identification (HPLC-DAD-MS)	Li et al. (2018)
Xinjiang jujube leaves	Flavonoids	Cleaned, dried in lyophilizer	342.39	11.56	50	1/43.95	70% Methanol	Ultrasound assisted extraction	DPPH; ABTS	-	-	-	Total Flavonoids (colorimetry); Identification (UPLC-ESI-MS)	Zhang et al. (2019)
Pomegranate peel	Phenolic compounds	Dried in a laboratory incubator with air circulation	300-600	15	RT	1/62	Water	Enzymatic extraction prior to HPE	DPPH	-	Well diffusion; MIC; MBC	-	Phenolic compounds (Folin-Ciocalteau); Identification (uHPLC)	Alexandre et al. (2019)
Ecliptae herba	Wedelolactone and isodemethyl-wedelolactone	Grounded	200	3	-	1/20	80% Methanol	Heat reflux	-	-	-	-	Identification (HPLC)	Zhao et al. (2019)
Yellow prickly pear peel	Bioactive compounds	Dried in a laboratory incubator with air circulation	300-600	5-30	RT	1/40	0-80% Ethanol	Soxhlet	DPPH; ABTS; FRAP	-	Well diffusion	-	Phenolic compounds (Folin-Ciocalteau); Total condensed tannins (Vanillin method); Total flavonoids (Dowd method); Total betalains (colorimetry); Total carotenoids (colorimetry)	Castro et al. (2019)
Tomato pulp	Flavonoids; Lycopene	Washed and blended	450	10	20	1/2	60% Hexane	Solid-liquid extraction	DPPH; FRAP	-	-	-	Phenolic compounds (Folin-Ciocalteau); Total Flavonoids (colorimetry); Identification (HPLC); Simulated gastrointestinal tract model	Briones-Labarca, Giovagnoli-Vicuña and Cañas-Sarazúa (2019)

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APPENDIX B

STANDARD CURVES

Table B1. Standard curves equations and linear correlation parameter for total phenolic compounds, total flavonoids, and antioxidant activities by ABTS, DPPH, FRAP and ORAC assays

Parameter	Equation	R^2
Total phenolic compounds [x = mg GAE/L | y = absorbance (750 nm)]		
Solvent – 0% ethanol	y = 0.0054x + 0.0591	0.994
	y = 0.0064x – 0.0126	0.995
	y = 0.0065x + 0.0198	0.994
Solvent – 35% ethanol	y = 0.0098x – 0.0345	0.997
Solvent – 70% ethanol	y = 0.0085x – 0.0781	0.993
Total flavonoids [x = µg rutin/mL | y = absorbance (415 nm)]	y = 0.0027x – 0.0131	0.995
	y = 0.0019x – 0.015	0.999
Antioxidant activity		
Radical cation ABTS scavenging activity [x = µg Trolox/mL | y = inhibition (%)*]	y = 0.7991x + 3.2231	0.995
	y = 1.0252 + 7.2126	0.980
Radical cation DPPH scavenging activity [x = µg Trolox/mL | y = inhibition (%)*]	y = 0.7369x + 2.6655	0.988
	y = 0.6675x + 0.7277	0.992
Ferric reduction antioxidant power [x = µg AIS/mL | y = absorbance (593 nm)]	y = 0.0030x – 0.0018	0.998
Oxygen radical absorbance capacity [x = µmol trolox | y = area (AUC)**]	y = 24044x – 0.9204	0.922

* $inhibition\ radical\ (\%) = 1 - \frac{absorbance\ (sample) - absorbance\ (blank)}{absorbance\ (control) - absorbance\ (blank)}\ x\ 100$

Where *absorbance (sample)* is the absorbance of each sample after mixture with the radical, *absorbance (blank)* is the absorbance of the mixture of distilled water and solvent, and *absorbance (control)* is the absorbance of the mixture of distilled water and the radical. The absorbances must be registered at 734 and 515 nm for ABTS and DPPH scavenging assays, respectively.

** $AUC = \frac{R1}{R1} + \frac{R2}{R1} + \frac{R3}{R1} + \cdots + \frac{Rn}{R1}$

Where AUC is the integral area below fluorescence decay curve of sample or standard minus the area below fluorescence decay curve of blank; R1 is the fluorescence read at the onset of the reaction and Rn is the last measurement.

Table B2. Calibration curves equations and linear correlation parameter for LC-MS/MS analysis

	Calibration curve [x = concentration (mg/mL) | y = area]	**Quantification limit (mg/L)**	**R^2**	**$t_{retention}$ (min)**	**$[M-H]^-$ experimental**	**MS/MS fragments**		
Total Phenolic compounds	*y* = 0.0054 x (gallic acid equivalents) – 0.0591	5.0	0.994	-	-	-	-	-
Neochlorogenic acid (3-caffeoylquinic acid)	*y* = 41461 x (chlorogenic acid equivalent) + 8260.7	0.25	0.997	7.1	353.0747	191.0491 (100)	179.0283 (76)	135.0398 (16)
Caftaric acid	*y* = 82077 x (caffeic acid equivalent) – 5643.9	0.25	0.998	7.3	311.0461	179.0374 (100)	149.0117 (46)	135.0477 (21)
5-*p*-Coumaroylquinic acid	*y* = 22757 x (*p*-coumaric acid equivalent) + 1986.2	0.25	0.997	8.2	337.0985	191.0586 (20)	163.0425 (100)	119.0523 (12)
Chlorogenic acid (5-caffeoylquinic acid)	*y* = 41461 x (chlorogenic acid) + 8260.7	0.25	0.997	8.4	353.0741	191.0488 (100)	161.0178 (2)	179.0277 (1)
Fertaric acid	*y* = 17500 x (ferulic acid equivalent) + 2998.2	0.25	0.992	9.3	325.0625	193.0525 (100)	149.0633 (5)	134.0394 (19)
4-*p*-Coumaroylquinic acid	*y* = 22757 x (*p*-coumaric acid equivalent) + 1986.2	0.25	0.997	9.8	337.0982	191.0583 (100)	173.0481 (10)	-
2-*O*-Caffeoylmalic acid	*y* = 82077 x (caffeic acid equivalent) – 5643.9	0.25	0.998	10.3	295.0512	179.0373 (38)	133.0168 (100)	-
***p*-Coumaric acid**	*y* = 22757 x (*p*-coumaric acid) + 1986.2	0.25	0.997	11.1	163.0397	163.0446 (15)	119.0547 (100)	-
Rutin	*y* = 39773 x (rutin) + 2773.2	0.25	0.993	11.7	609.1679	300.0332 (100)	-	-
***p*-Coumaroylmalic acid Isomer 1**	*y* = 22757 x (*p*-coumaric acid equivalent) + 1986.2	0.25	0.997	11.9	279.0581	163.0449 (100)	133.0183 (5)	119.0549 (16)
***p*-Coumaroylmalic acid Isomer 2**	*y* = 22757 x (*p*-coumaric acid equivalent) + 1986.2	0.25	0.997	12.3	279.0558	163.0447 (64)	133.0190 (100)	119.0544 (18)
Isoferulic acid	*y* = 17500 x (ferulic acid equivalent) + 2998.2	0.25	0.992	12.5	193.0531	134.0410 (100)	178.0306 (2)	-

Table B3. Standard curves equations and linear correlation parameter for HPLC analysis

Parameter	**Calibration curve** [x = concentration (mg/mL) | y = area]	**Quantification limit (mg/mL)**	**R^2**
Chlorogenic acid (and equivalents)	$y = 6x10^7x + 369926$	0.016	0.9998
Caffeic acid (and equivalents)	$y = 1x10^8x + 485096$	0.008	0.9981
Rutin (and equivalents)	$y = 3x10^7x + 132541$	0.008	0.9984
Rosmarinic acid (and equivalents)	$y = 6x10^7x + 204735$	0.008	0.9983
Rutin (and equivalents)	$y = 1x10^8x + 213534$	0.008	0.9990
Isoferulic acid (and equivalents)	$y = 1x10^8x - 556255$	0.008	0.9994
Sinapic acid (and equivalents)	$y = 1x10^8x - 555914$	0.008	0.9993
p-coumaric (and equivalents)	$y = 1x10^8x + 213534$	0.004	0.9990
Ferulic acid (and equivalents)	$y = 1x10^8x + 478721$	0.008	0.9981

APPENDIX C

SUPPLEMENTARY TESTS

APPENDIX C

C1 – Mass/volume ratio determination for stinging nettle leaves (fresh sample)

Generally, it was observed that the ratio of mass of herb (g) to volume of solvent (mL) greatly affected the extraction of total phenolic compounds (**Figure C1**), total flavonoids (**Figure C2)**, and pigments (**Figure C3** and **C4)**, as also the antioxidant activity of the final extracts (**Figure C5** and **C6)**, and the general extraction yield (**Figure C7**). For so, it was decided to choose the ratio 1/20 for performing the extracts to which the biological activities were studied (**Chapter V, section 5.1**).

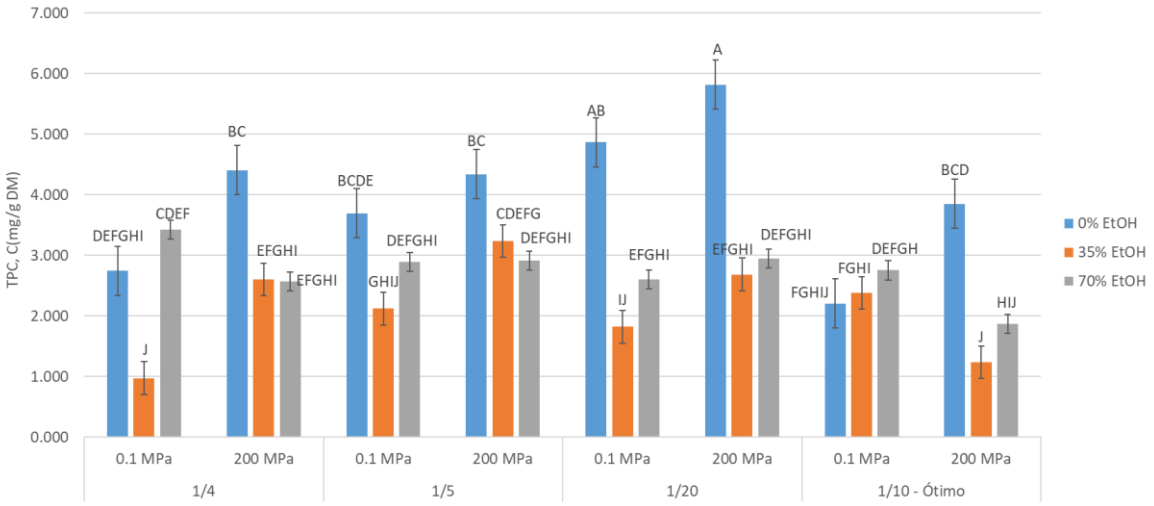


Figure C1. Ratio study concerning total phenolic compounds extracted from stinging nettle leaves

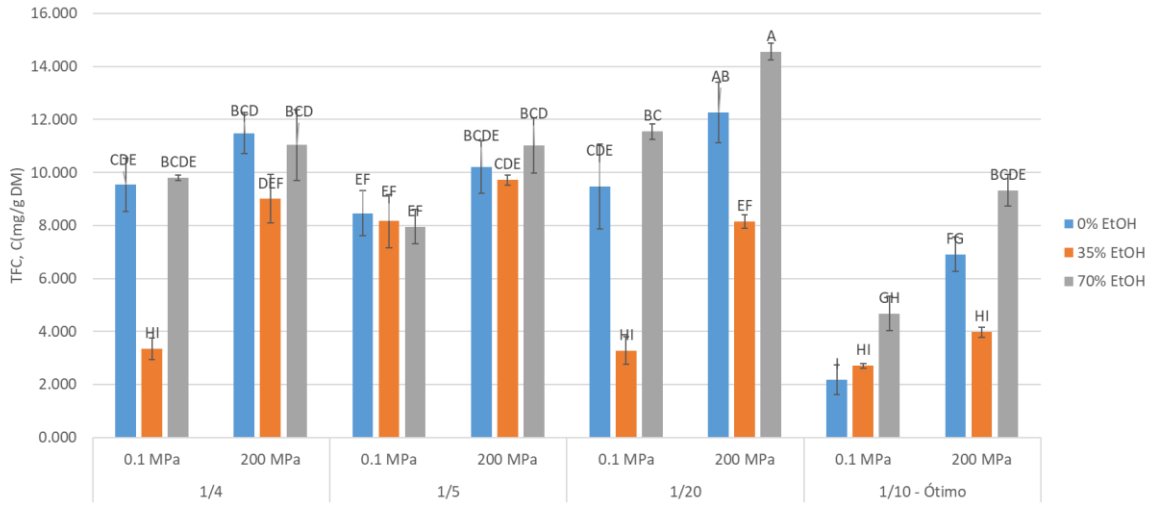


Figure C2. Ratio study concerning total flavonoids extracted from stinging nettle leaves

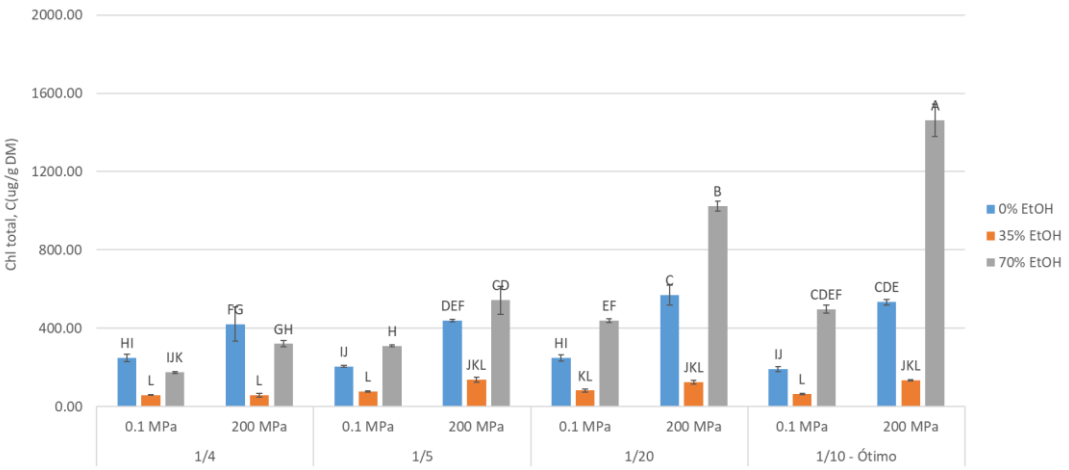


Figure C3. Ratio study concerning total chlorophylls extracted from stinging nettle leaves

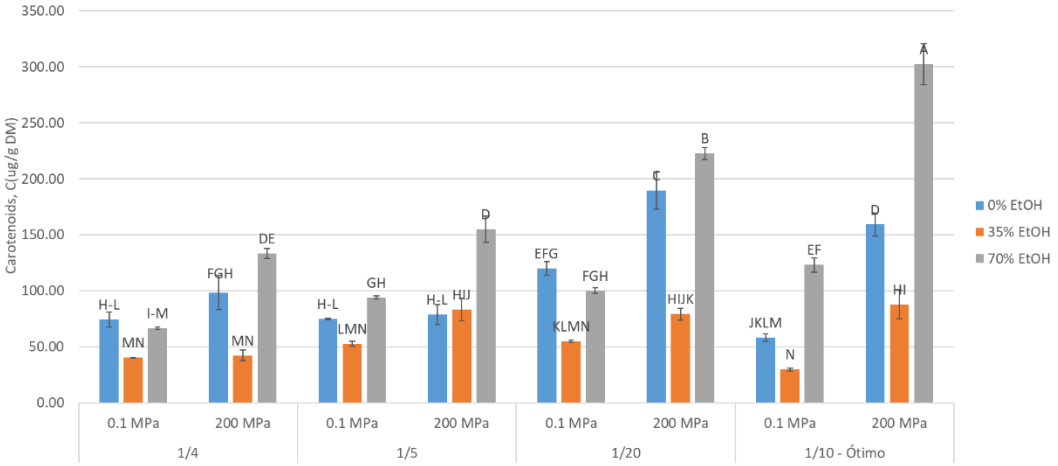


Figure C4. Ratio study concerning total carotenoids extracted from stinging nettle leaves

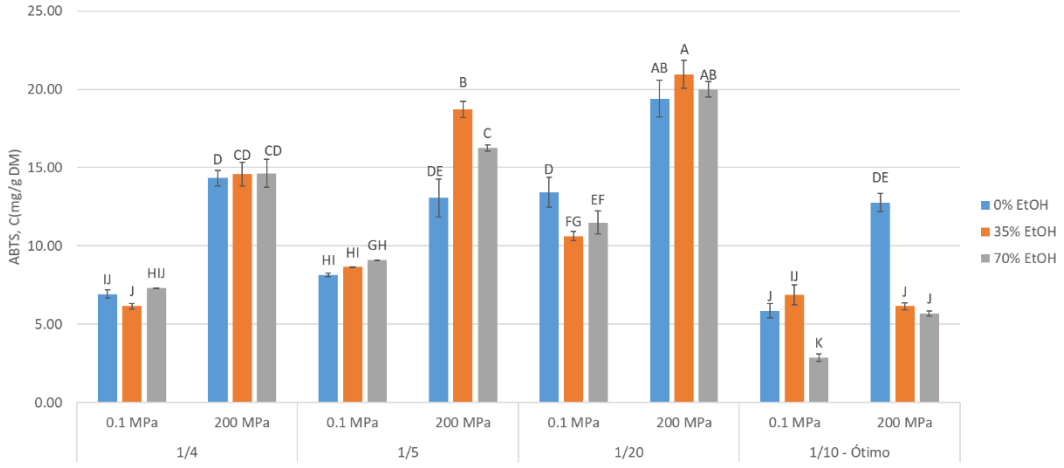


Figure C5. Ratio study concerning antioxidant activity (ABTS assay) of extracts from stinging nettle leaves

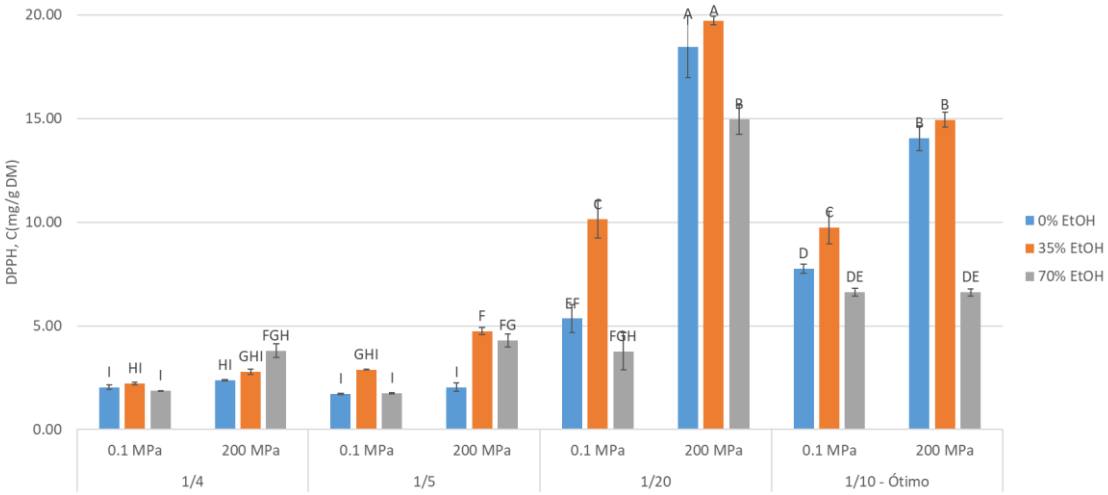


Figure C6. Ratio study concerning antioxidant activity (DPPH assay) of extracts from stinging nettle leaves

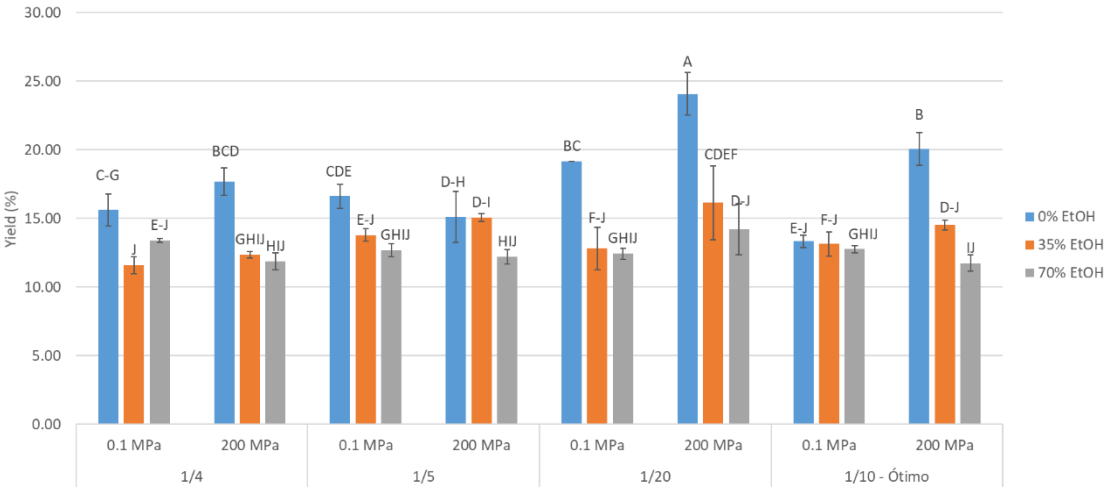


Figure C7. Ratio study concerning extraction yield from stinging nettle leaves

C2 – Mass/volume ratio determination for winter savory leaves (dried sample)

For winter savory, the study of the extraction ratio was performed before the optimization study, and for so, only the control (0.1 MPa) and the intermediate pressure (350 MPa) were studied for 10 min, to verify were the saturation of the solvent with the herb would occur. And only two responses were studies, the total phenolic compounds (**Figure C8)** and antioxidant activity by ABTS assay (**Figure C9**). Considering these results, it was decided to choose the ratio 1/10 for performing the extracts to which the biological activities were studied (**Chapter V, section 5.2**).

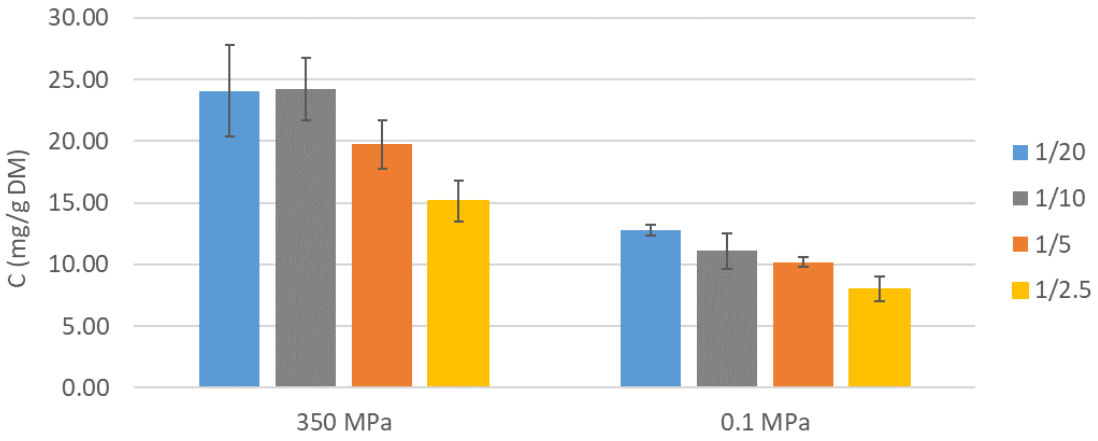


Figure C8. Ratio study concerning total phenolic compounds extracted from winter savory leaves

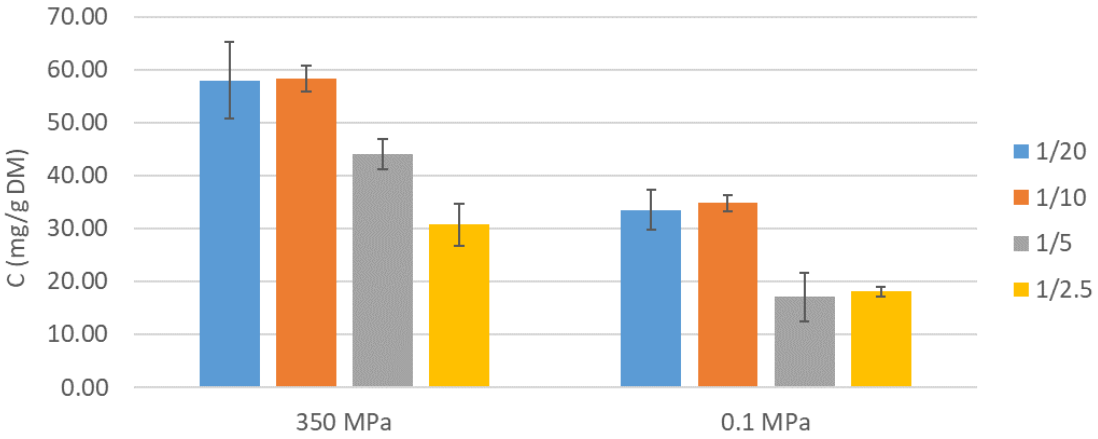


Figure C9. Ratio study concerning antioxidant activity (ABTS assay) of extracts from winter savory leaves

C3 – Need to use Ultraturrax to sample homogenization before extraction process

A preliminary test was performed in order to verify the need to use an Ultraturrax to homogenize the initial sample. The samples at left on the **Figure C10** show the samples after HPE with a pre-homogenization using an Ultraturrax equipment, while the samples at the right show the extracts after HPE with no pre-treatment with an Ultraturrax equipment.

(a)

(b)

(c)

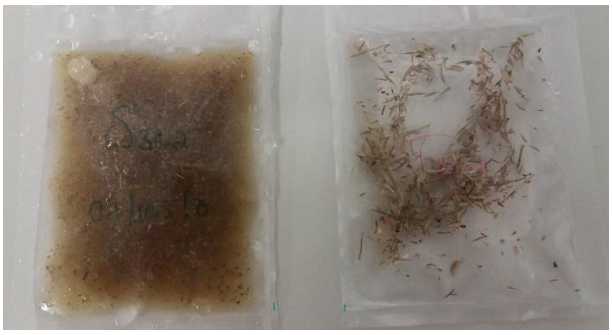


Figure C10. Comparison between extracts submitted (at left) and not submitted (at right) to an Ultraturrax homogenization. (a) aqueous extracts, (b) 35% ethanol extracts, (c) 70% ethanol extracts

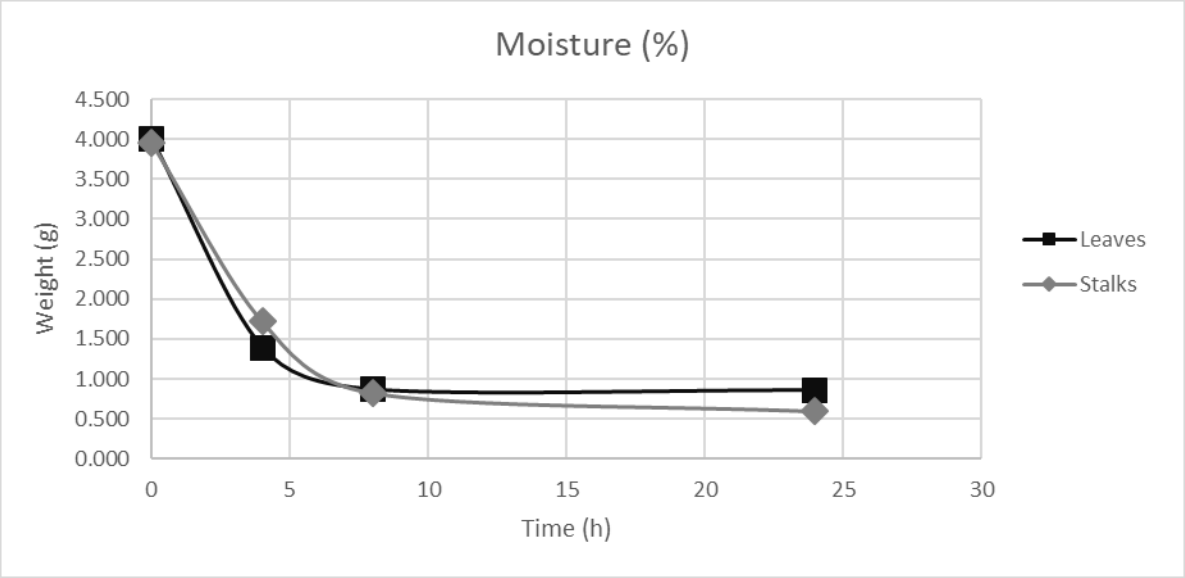
C4 – Moisture analysis of stinging nettle leaves (fresh sample)

A moisture analysis on fresh nettle leaves was performed in order to access the amount of herb to weight in order to have a mass of fresh herb equivalent to the mass of dried herb in each extraction.

For so, 3.0 g of nettle from each part (leaves and stalks) was weighed and placed in a drying oven at 103 ºC for 4 h. Then, each sample was taken out from the drying oven and then kept in desiccators for 30 min to reach the room temperature. After being weighed, samples were put again in drying oven for 1 h to keep drying till a constant weight. The moisture content of samples was calculated using the following formula:

Moisture (%) = $\frac{m1-m2}{m1}$ x 100,

where *m1* is initial weigh of sample, *m2* is final weigh of sample.



The results obtained demonstrated that stinging nettle leaves had a moisture of 78.64 ± 2.43%, while the stalks had a value of 84.93 ± 0.74%.

C5 – pH analysis

Table C1. pH analysis of stinging nettle optimized extracts. Comparison between fresh sample and sample after frozen storage. Different letters indicate significant differences (p>0.05) between pH of the fresh sample and the correspondent frozen stored sample (analysis by row)

Extract	pH (fresh sample)	pH (thawed sample)
200 MPa / 10.6 min / 0% ethanol	7.85 ± 0.04 a	7.88 ± 0.01 a
0.1 MPa / 10.6 min / 0% ethanol	7.93 ± 0.11 a	7.01 ± 0.02 b
500 MPa / 10.0 min / 25% ethanol	7.18 ± 0.02 a	7.19 ± 0.01 a
0.1 MPa / 10.0 min / 25% ethanol	7.03 ± 0.02 a	7.05 ± 0.01 a
200 MPa / 10.6 min / 35% ethanol	7.19 ± 0.04 a	7.20 ± 0.02 a
0.1 MPa / 10.6 min / 35% ethanol	7.19 ± 0.01 a	7.21 ± 0.02 a
200 MPa / 10.6 min / 70% ethanol	6.96 ± 0.03 ab	7.01 ± 0.02 b
0.1 MPa / 10.6 min / 70% ethanol	7.03 ± 0.02 a	7.03 ± 0.02 a

Table C2. pH analysis of winter savory optimized extracts. Comparison between fresh sample and sample after frozen storage. Different letters indicate significant differences (p>0.05) between pH of the fresh sample and the correspondent frozen stored sample (analysis by row)

Extract	pH (fresh sample)	pH (thawed sample)
200 MPa / 10.6 min / 0% ethanol	6.28 ± 0.02 a	6.32 ± 0.03 a
0.1 MPa / 10.6 min / 0% ethanol	6.14 ± 0.01 a	6.11 ± 0.04 a
200 MPa / 10.6 min / 35% ethanol	6.07 ± 0.02 a	6.09 ± 0.01 a
0.1 MPa / 10.6 min / 35% ethanol	6.29 ± 0.01 a	6.30 ± 0.01 a
200 MPa / 10.6 min / 70% ethanol	6.00 ± 0.02 ab	6.02 ± 0.02 a
0.1 MPa / 10.6 min / 70% ethanol	6.03 ± 0.02 ab	6.05 ± 0.01 a

C6 – Decision-making process for definition of 'general optimal conditions'

C6.1 – Stinging nettle

The following tables show the decision-making process which was performed in order to cluster all the responses into the fewer extracts possible. In **Table C3**, it is possible to see the optimal predicted conditions for each model for the respective response, as also the optimal predicted values (marked at blue); whereas the remaining values of each row are the predicted response-value for those optimal conditions (for example, by using the condition set of 200 MPa, 10.6 min, 0% ethanol, one would obtain a maximum total phenolic compounds (TPC) of 4.00 mg/g, a maximum of flavonoids (TFC) of 8.62 mg/g, a maximum of total chlorophylls (ChlT) of 701.72 µg/g, etc.).

Table C3. Optimal conditions predicted by each individual model

	Optimal conditions predicted by each individual model			**Optimal values predicted by each individual model (cell marked at blue background)**								
Response	**Pressure**	**time**	**Solvent**	**TPC**	**TFC**	**ABTS**	**DPPH**	**ChlA**	**ChlB**	**ChlT**	**Carotenoids**	**Yield**
TPC	200	10.6	0	4.00	8.62	11.70	15.72	194.71	517.51	701.72	287.56	20.16
				0%	-8%	0%	-16%	-71%	-12%	-45%	-8%	-2%
TFC	200	10.6	70	2.87	9.35	6.04	13.86	670.62	586.90	1269.70	311.06	10.96
				-28%	0%	-48%	-26%	0%	0%	0%	0%	-47%
ABTS	200	10.6	0	4.00	8.62	11.70	15.73	194.71	517.50	701.70	287.56	20.16
				0%	-8%	0%	-16%	-71%	-12%	-45%	-8%	-2%
DPPH	500	9.8	26.9	3.38	5.70	10.51	18.63	32.63	300.40	237.00	218.67	15.19
				-15%	-39%	-10%	0%	-95%	-49%	-81%	-30%	-26%
Chl	200	10.4	70	2.87	9.35	6.04	13.87	670.78	586.94	1269.81	311.16	10.93
				-28%	0%	-48%	-26%	0%	0%	0%	0%	-47%
Carotenoids	200	10.2	70	2.87	9.34	6.03	13.87	670.24	586.80	1269.70	311.16	10.90
				-28%	0%	-48%	-26%	0%	0%	0%	0%	-47%
Yield	200	15.6	0	3.57	7.69	10.61	14.91	180.11	458.10	634.90	252.34	20.6
				-11%	-18%	-9%	-20%	-73%	-22%	-50%	-19%	0%

After, the predicted values so obtained were compared (by percentage) with their own predicted individual optimum values and the different response variables were clustered (considering a difference <10%) for each ethanol concentration studied (0%, 35%, and 70% ethanol). This way, four sets of general experimental conditions were obtained (**Table C4**).

Table C4. Clustering of the several responses into 'general optimal conditions'

	Optimal conditions predicted by each individual model			Optimal values predicted by each individual model (cell marked at blue background)								
Response	**Pressure**	**time**	**Solvent**	**TPC**	**TFC**	**ABTS**	**DPPH**	**ChlA**	**ChlB**	**ChlT**	**Carotenoids**	**Yield**
General (0% ethanol)	200	11.6	0	3.99	8.58	11.66	-	-	-	-	-	20.29
TPC, TFC, ABTS and Yield				0%	-8%	0%	-	-	-	-	-	-1%
General (25% ethanol)	500	10	70	-	-	-	18.628	-	-	-	-	-
DPPH				-	-	-	0%	-	-	-	-	-
General (35% ethanol)	200	11.6	35	3.99	8.58	11.66	-	-	-	-	-	20.29
TPC, TFC, ABTS e Yield				0%	-8%	0%	-	-	-	-	-	-1%
General (70% ethanol)	200	10.2	70	-	-	-	-	670.25	586.85	1269.69	311.16	-
Chl + Carot				-	-	-	-	0%	0%	0%	0%	-

C6.2 – Winter savory

For winter savory extracts, the decision-making process followed the same steps mentioned above for stinging nettle.

Table C5. Optimal conditions predicted by each individual model and clustering of the several responses into 'general optimal conditions'

	Optimal conditions predicted by each individual model			Optimal values predicted by each individual model (cell marked at blue background)									
Response	**Pressure**	**time**	**Solvent**	**TPC**	**TFC**	**ABTS**	**DPPH**	**FRAP**	**ChlA**	**ChlB**	**ChlT**	**Carot.**	**Yield**
TPC	357.6	20	19.5	25.95	15.39	87.04	53.86	357.53	-1.90	58.77	64.32	59.75	16.72
				0%	-7%	-3%	-15%	-3%	-101%	-59%	-84%	-44%	-12%
TFC	351.5	1	0	23.68	16.61	64.32	43.60	308.59	37.03	110.27	164.93	76.38	18.93
				-9%	0%	-29%	-31%	-16%	-86%	-23%	-59%	-28%	0%
ABTS	360.6	20	31.8	25.57	15.03	90.12	51.81	366.14	5.82	37.41	47.55	54.99	16.07
				-1%	-10%	0%	-18%	-1%	-98%	-74%	-88%	-48%	-15%
DPPH	500	20	0	22.21	13.31	61.75	63.51	283.10	68.48	143.26	213.63	77.56	17.49
				-14%	-20%	-31%	0%	-23%	-75%	0%	-47%	-27%	-8%
FRAP	327.3	20	36	25.14	14.84	89.30	50.26	368.17	14.17	34.49	51.56	53.82	15.54
				-3%	-11%	-1%	-21%	0%	-95%	-76%	-87%	-49%	-18%
Chl	500	1	70	15.55	11.96	50.57	22.66	294.46	272.48	143.24	402.88	100.38	17.82
				-40%	-28%	-44%	-64%	-20%	0%	0%	0%	-6%	-6%
Carot.	500	20	70	16.87	11.30	55.13	55.31	310.26	243.07	120.26	351.56	106.29	16.70
				-35%	-32%	-39%	-13%	-16%	-11%	-16%	-13%	0%	-12%
Yield	350	1	0										18.93
				-	-	-	-	-	-	-	-	-	0%

General (0% ethanol)	500	20	0	22.21	13.31	61.75	63.51	283.10	68.48	143.26	213.63	77.56	17.49
DPPH				-14%	-20%	-31%	0%	-23%	-75%	0%	-47%	-27%	-8%
General (35% ethanol)	348.5	20	35	25.34	14.94	89.79	51.03	367.60	12.18	35.28	51.07	54.48	15.79
TPC + ABTS + FRAP + TFC				-2%	-10%	0%	-20%	0%	-96%	-75%	-87%	-49%	-17%
General (70% ethanol)	500	1	70	15.55	11.96	50.57	22.66	294.46	272.48	143.24	402.88	100.38	17.82
Chl + Carot				-40%	-28%	-44%	-64%	-20%	0%	0%	0%	-6%	-6%

APPENDIX D

STATISTICAL ANALYSIS | RESPONSE SURFACE METHODOLOGY

D1 – Statistical analyses and model's construction

The statistical analyses and model's construction were performed using a Minitab Statistical Software (version 17.0, Minitab Ltd., Coventry, United Kingdom). A response surface methodology (RSM) was used to analyse the relationships between the independent variables (individual effects and possible interactions), as well as to find the optimum extraction conditions for each studied response. The experimental design followed a central composite face-centred design (CCD). After defining the experimental design with a total of 14 random runs and an additional 6 central points with triplicates, the total experimental runs summed up to 60 points. In this Appendix, and since the line of reasoning was the same for all the models and response variables, it is only shown as example, the statistical analysis and model construction for total phenolic compounds extraction from winter savory leaves.

1. **Residual plots.** The first step to verify if the response surface regression fits well to the experimental data is the analysis of the residual plots (**Figure D1**). A residual plot is a graph that is used to examine the goodness-of-fit in regression and ANOVA. If the ordinary least squares assumptions are satisfied, then ordinary least squares regression will produce unbiased coefficient estimates with the minimum variance.

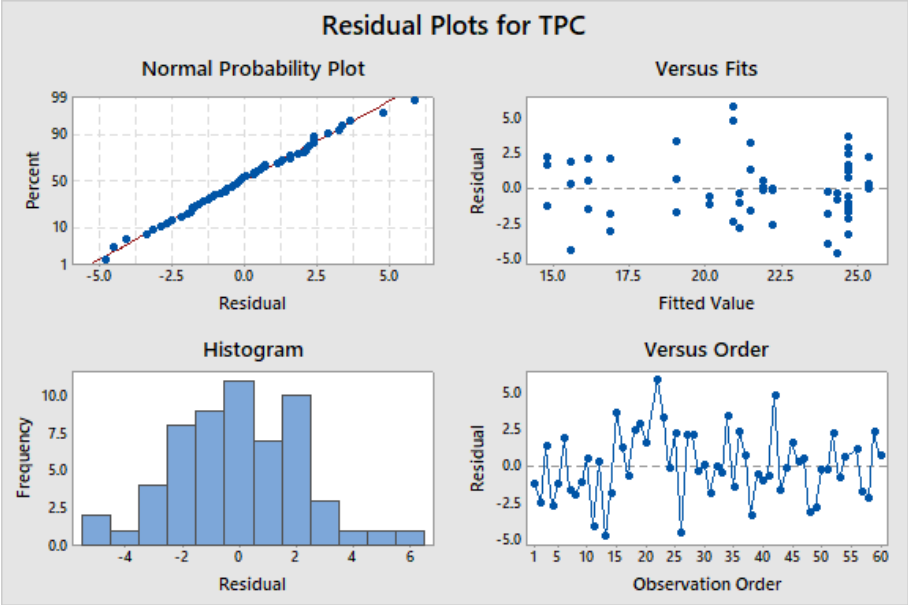


Figure D1. Residual plots for total phenolic compounds

The normal probability plot helps to verify if the residuals are normally distributed (the dots should follow the probability line - Anderson-Darling test). The histogram determines whether the data are skewed or whether outliers exist in the data and should

present the shape of a bell (or approximately). This graph is essentially to determine if the experimental data can be submitted to ANOVA analysis, since it needs the assumption that the residuals (and not the data itself) follow a normal distribution. The plot of 'Residuals versus fits' helps to verify the assumption that the residuals have a constant variance (it should show a random pattern of residuals on both sides of zero). Finally, the 'Residuals versus order' is used to verify the assumption that the residuals are uncorrelated with each other (it should show a random pattern, indicating that there is an independence between the residuals; if some systematic pattern is observed in this graph, it is probable that some "extra" variable had influence on the experiment).

2. **Analysis of variance.** After verifying the residuals behaviour, a table of ANOVA is generated, which helps to see if there are interactions between the variables, and to define which of the terms (linear, square, interactions) have the major contribution to the models construction, the higher impact (F-value) and if they are significant to the model or not (p-value) (**Table D1**).

Table D1. Analysis of variance (ANOVA)

Source	DF	Seq SS	Contribut.	Adj SS	Adj MS	F-value	p-value
Model	9	821.62	69.60%	821.616	91.291	12.72	0.000
Linear	3	208.79	17.69%	208.789	69.596	9.70	0.000
Pressure	1	14.83	1.26%	14.829	14.829	2.07	0.157
Time	1	29.21	2.47%	29.214	29.214	4.07	0.049
Solvent	1	164.75	13.96%	164.745	164.745	22.95	0.000
Square	3	564.51	47.82%	564.507	188.169	26.21	0.000
Pressure*Pressure	1	456.86	38.70%	97.104	97.104	13.53	0.001
Time*Time	1	19.98	1.69%	0.400	0.400	0.06	0.814
Solvent*Solvent	1	87.67	7.43%	87.672	87.672	12.21	0.001
Interaction between 2 factors	3	48.32	4.09%	48.321	16.107	2.24	0.095
Pressure*Time	1	25.49	2.16%	25.492	25.492	3.55	0.065
Pressure*Solvent	1	21.60	1.83%	21.601	21.601	3.01	0.089
Time*Solvent	1	1.23	0.10%	1.228	1.228	0.017	0.681
Error	50	358.92	30.40%	358.918	7.178	-	-
Lack-of-fit	5	62.44	5.29%	62.441	12.488	1.90	0.114
Pure error	45	296.48	25.11%	296.477	6.588	-	-
Total	59	1180.53	100.0%	-	-	-	-

Model summary:

S = 2.67925 | R-sq = 69.60% | R-sq(adj) = 64.12% | PRESS = 558.883 | R-sq(pred) = 52.66%

Regression equation in uncoded units:

TPC = -2.73 + 0.1253 Pressure + 0.432 Time + 0.1897 Solvent - 0.000152 Pressure*Pressure

- 0.0024 Time*Time - 0.002661 Solvent*Solvent - 0.000723 Pressure*Time

- 0.000181 Pressure*Solvent - 0.00068 Time*Solvent

Table D2. Fits and diagnostics for unusual observations

Obs	TPC	Fit	SE fit	95% CI	Resid	Std resid	Del resid	HI
21	10.430	16.526	1.378	(13.758; 19.293)	-6.096	-2.65	-2.83	0.264394
22	26.731	21.890	1.378	(19.123; 24.657)	4.841	2.11	2.18	0.264394
26	11.014	15.758	1.378	(12.991; 18.526)	-4.744	-2.06	-2.14	0.264394
55	31.079	24.988	0.532	(23.920; 26.056)	6.091	2.32	2.43	0.039394

Considering the values of p-value in **Table D1** it is possible to conclude that the terms linear 'pressure', square 'time', and all the three interactions are not-significative ($p>0.05$). This is also observed in the section 'Model summary', since all the regression coefficients (R^2, R^2 (adjusted), and R^2 (predicted)) are below 75%, indicating that this model does not fit well the associated experimental data. Also in **Table D2**, the unusual observations are presented, indicating the actual experimental values ('TPC') and the predicted values by a well-fitted model ('Fit').

For so, a step wise procedure was conducted (which was responsible to remove/add terms to the model for the purpose of identifying a useful subset of the terms; by default, this procedure starts with an empty model and then adds or removes a term for each step according to an alpha value). The stepwise procedure added terms during the procedure in order to maintain a hierarchical (in a hierarchical model, all lower-order terms that comprise the higher-order terms also appear in the model) model at each step. Response surface design models must be hierarchical in order to produce an equation in natural units.

After that, another ANOVA table (**Table D3**) was generated, as also as another model summary and another regression equation.

Table D3. Analysis of variance (ANOVA) after performing a stepwise procedure

Source	DF	Seq SS	Contribut.	Adj SS	Adj MS	F-value	p-value
Model	5	678.275	70.21%	678.275	135.655	24.51	0.000
Linear	3	239.258	24.77%	228.101	76.034	13.74	0.000
Pressure	1	3.424	0.35%	4.174	1.174	0.75	0.389
Time	1	12.241	1.27%	12.731	12.731	2.30	0.135
Solvent	1	223.592	23.14%	205.341	205.341	37.10	0.000
Square	2	439.017	45.44%	439.017	219.509	39.66	0.000
Pressure*Pressure	1	352.987	36.54%	96.084	96.084	17.36	0.000
Solvent*Solvent	1	86.030	8.91%	86.030	86.030	15.54	0.000
Error	52	287.798	29.79%	287.798	5.535	-	-
Lack-of-fit	9	75.687	7.83%	75.687	8.410	1.70	0.117
Pure error	43	212.111	21.96%	212.111	4.933	-	-
Total	57	966.073	100.0%	-	-	-	-

Stepwise selection of terms:

α to enter = 0.15; α to remove = 0.15 (default values)

Model summary:

S = 2.35257 | R-sq = 80.21% | R-sq(adj) = 77.35% | PRESS = 366.301 | R-sq(pred) = 76.08%

Regression equation in uncoded units:

TPC = 5.42 + 0.1014 Pressure + 0.0699 Time + 0.0957 Solvent

- 0.000141 Pressure*Pressure - 0.002455 Solvent*Solvent

Table D4. Fits and diagnostics for unusual observations after a stepwise procedure

Obs	TPC	Fit	SE fit	95% CI	Resid	Std resid	Del resid	HI
13	19.561	24.340	0.869	(22.595; 26.084)	-4.779	-2.19	-2.27	0.136512
26	11.014	15.545	0.872	(13.794; 17.296)	-4.531	-2.07	-2.14	0.137538
42	25.670	20.878	0.891	(19.090; 22.665)	4.792	2.20	2.29	0.143338

One could keep endlessly deleting the unusual observations (**Table D4**) or keeping performing more experimental runs in order to replace these values, but since the model summary, residuals, and ANOVA values are according to the expected, the model is admitted valid as it is.

3. **Analysis of interactions.** In order to visualize the effect of interactions, two graphs can be generated: the 'main effects plot' and the 'interaction plot'. The main effects plot is used when there are multiple factors (in this case, pressure, time, and solvent). The points in the plot are the raw data means of the response variable at the various levels of each factor. In **Figure D2** it is corroborated the data gathered in **Table D3**: 'pressure' and 'solvent' terms follow quadratic behaviours, while 'time' term follows a linear behaviour.

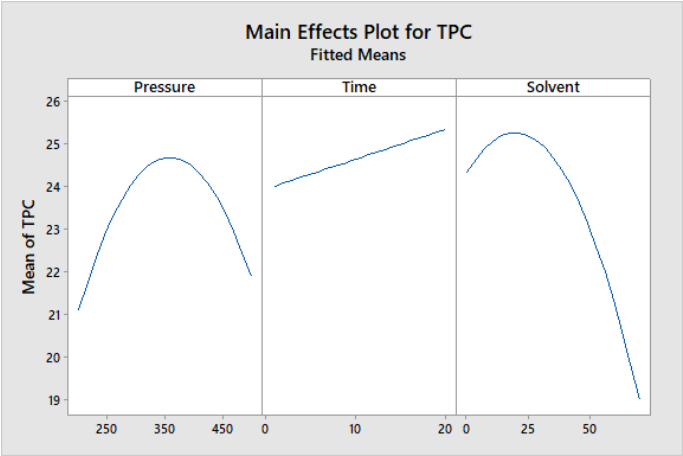


Figure D2. Main effects plot generated for total phenolic compounds model

Concerning the interactions plot, as was also observed in **Table D3** and in the regression equation, no interactions are observed between the factors, and for so, the note 'There are no valid interactions to plot' appears in the program. Nevertheless, an example is presented in **Figure D3**, of this type of plot, where a slight interaction is observed between 'solvent' and 'pressure' terms (since the respective plots overlap; the more unparalleled the lines, the stronger the interaction) – this can also be observed in the regression equation obtained for this particular response (see **Table 4.5**, from **Chapter IV**, **section 4.2**).

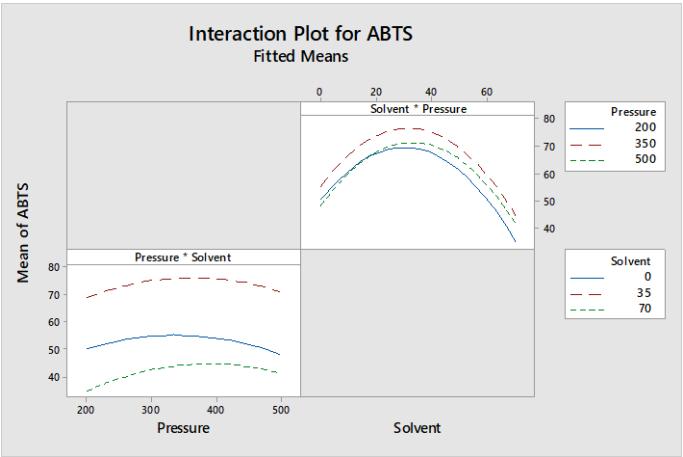


Figure D3. Interaction plot generated for ABTS radical scavenging assay

4. **Surface plots design**. After assuring that the model is a good-fit to the experimental data, one can now generate either a response surface plot of a contour plot. Surface plots show how the fitted response relates to two continuous variables. A surface plot displays the three-dimensional relationship in two dimensions, with the variables on the x- and y-scales, and the response (z) variable represented by a smooth surface (**Figure D4**).

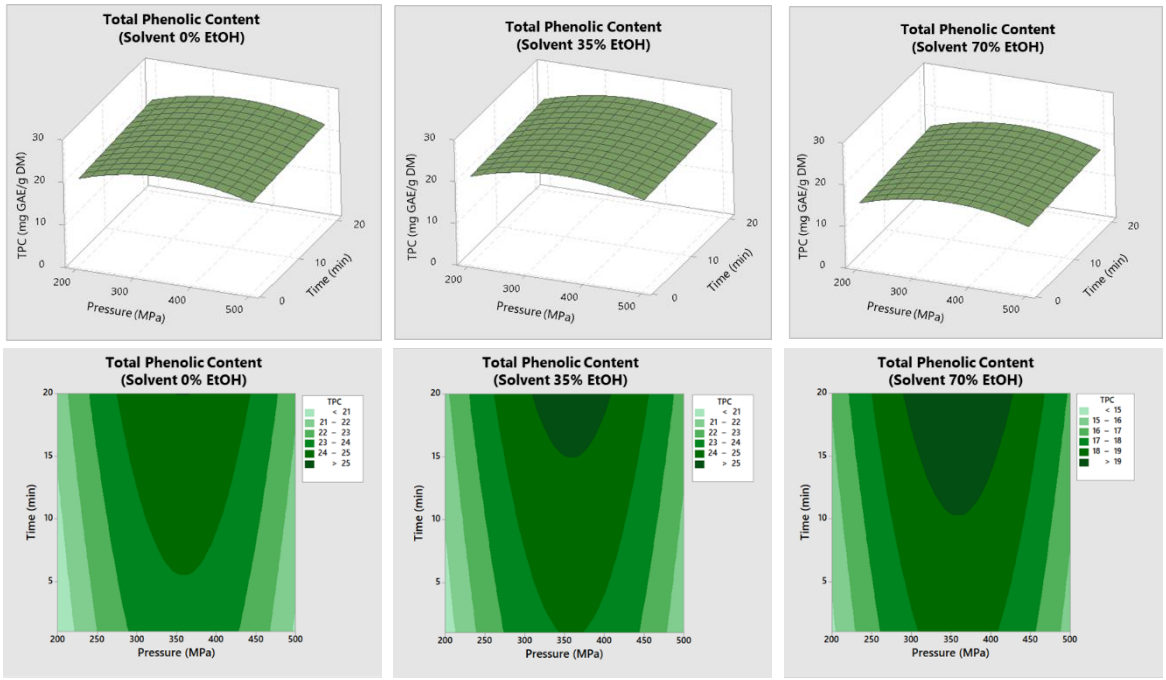


Figure D4. Response surface plots (first row) and contour pots (second row) for total phenolic compounds extraction from winter savory leaves

5. Response optimizer. Response optimization identifies the combination of input variable settings (process conditions) that jointly optimize a single response (**Figure D5**) or a set of responses (**Figure D6**).

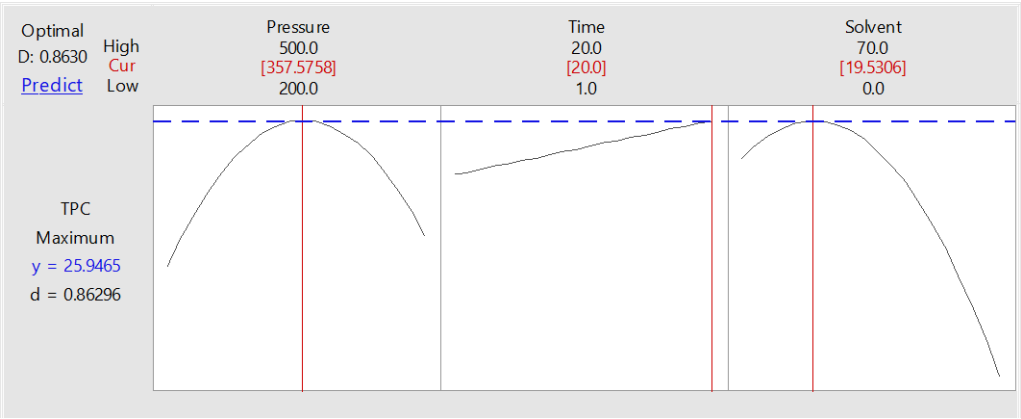


Figure D5. Optimization plot for total phenolic compounds extracted from winter savory leaves

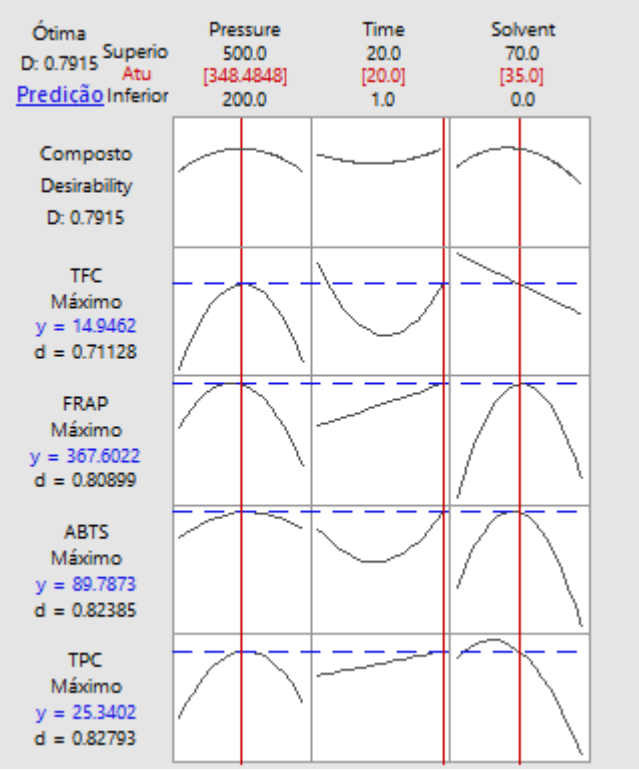
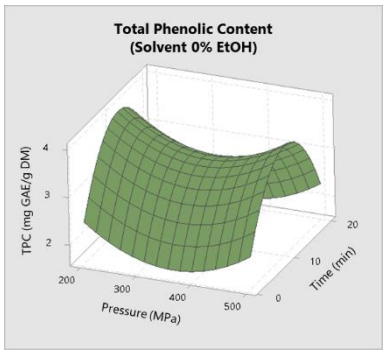


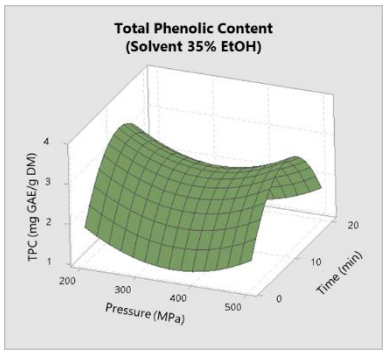
Figure D6. Optimization plot for a set of responses (total phenolics, total flavonoids, and antioxidant activity (by FRAP and ABTS assays)

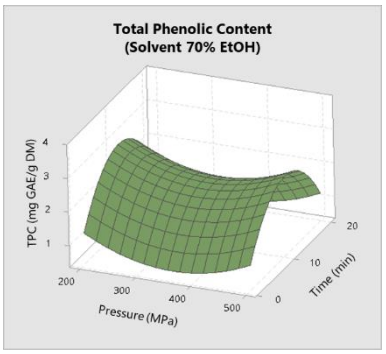
Joint optimization must satisfy the requirements for all the responses in the set, which is measured by the desirability, D, which may present a maximum value of 1.0. In **Figure D6**, all the desirability values are above 0.70, indicating that the optimal predicted values (y) for the optimal process conditions (indicated at red), are similar to the values predicted if the optimization plot were drawn for each response at a time.

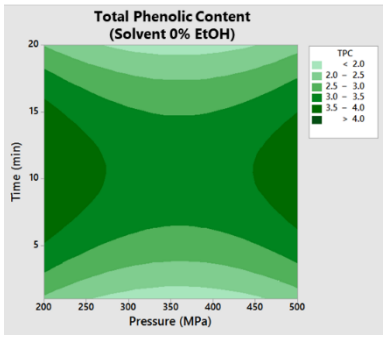
D2 – Response surface graphs for stinging nettle extraction

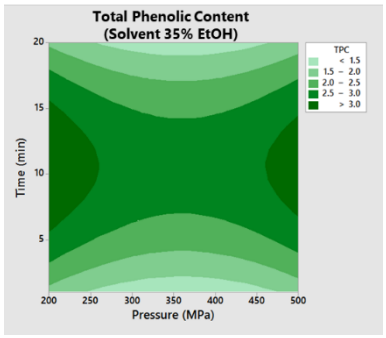
Total phenolic compounds

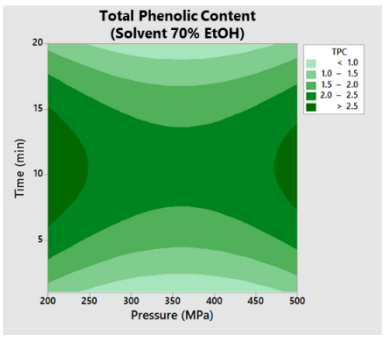




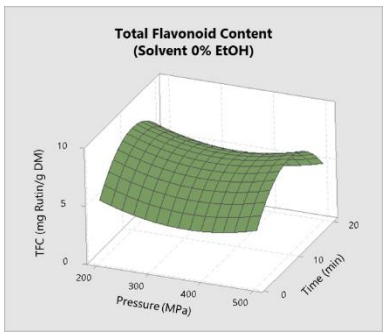


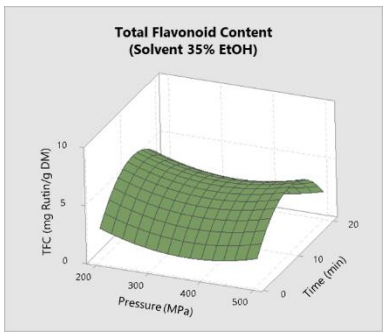


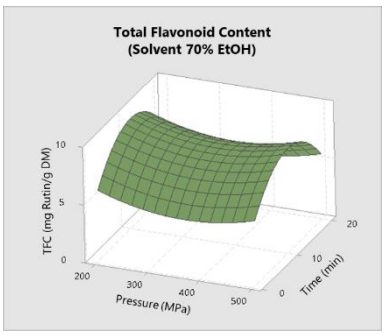


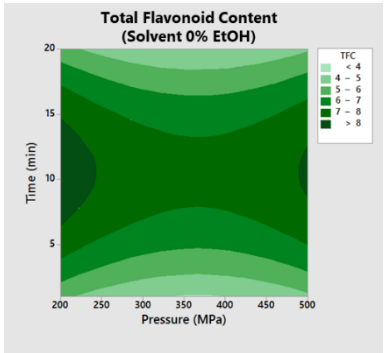


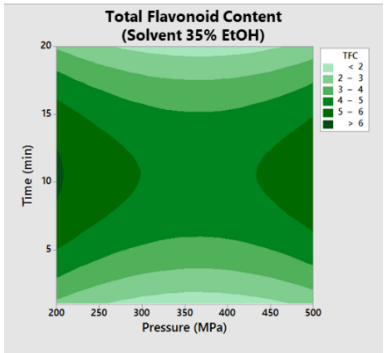
Total flavonoids

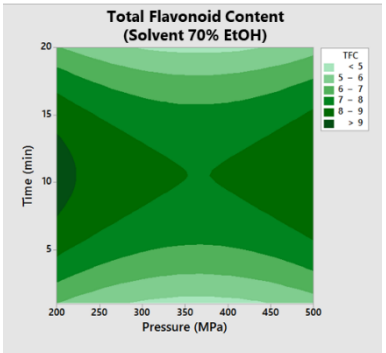




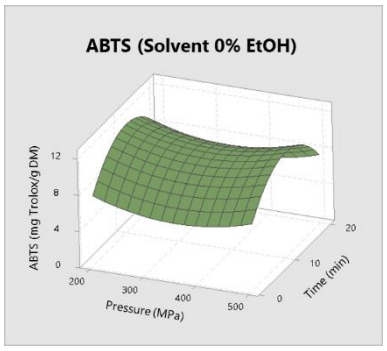


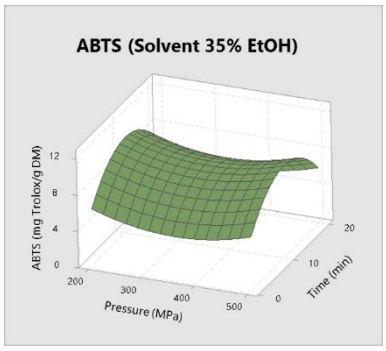


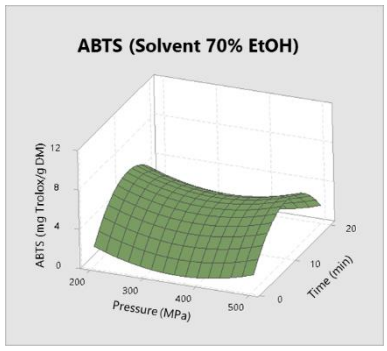


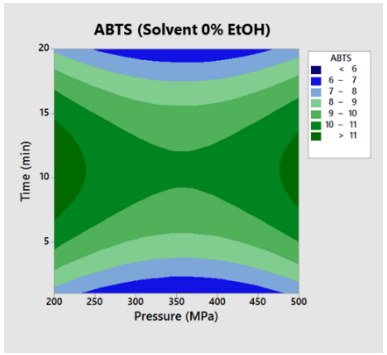


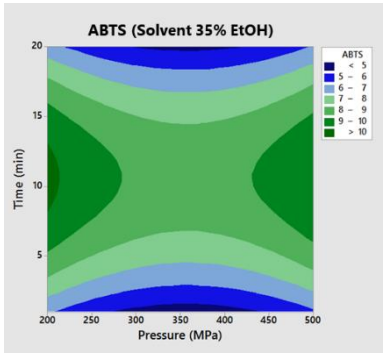
Antioxidant activity by ABTS assay

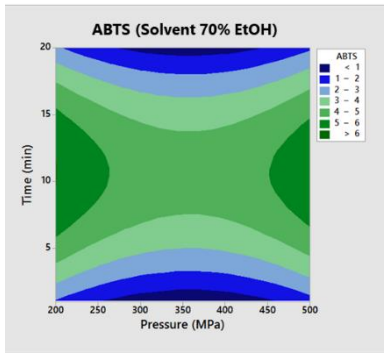




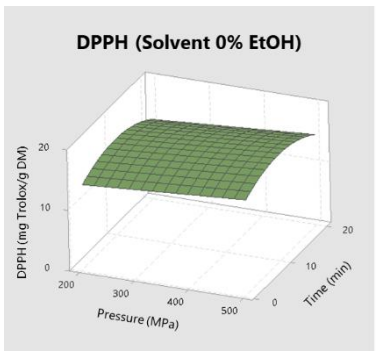


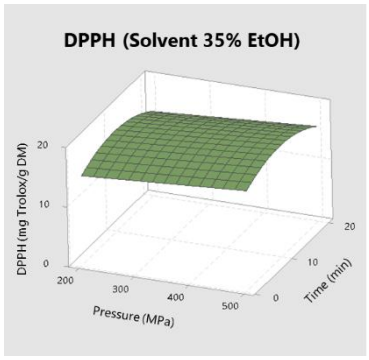


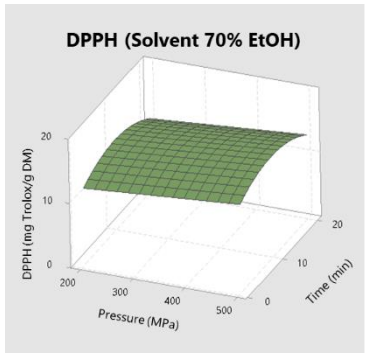


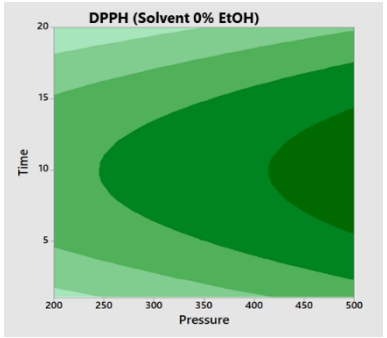


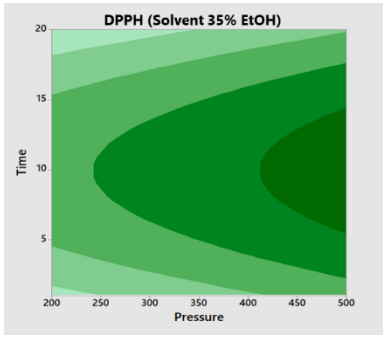
Antioxidant activity by DPPH assay

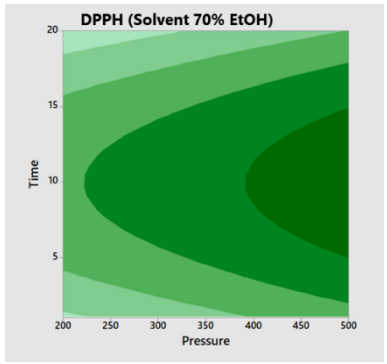




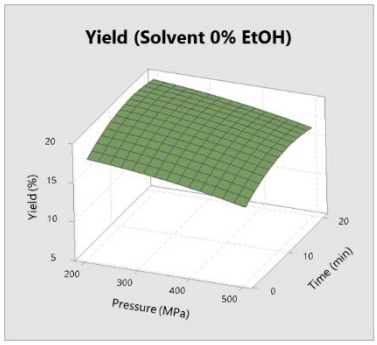


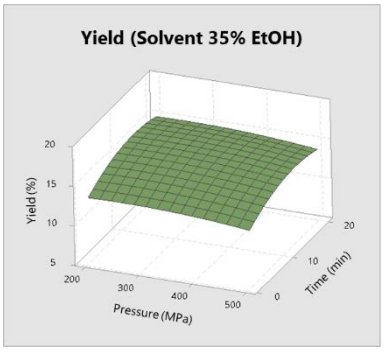


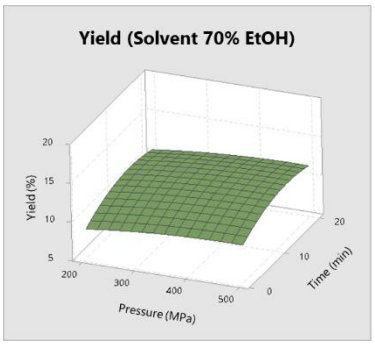


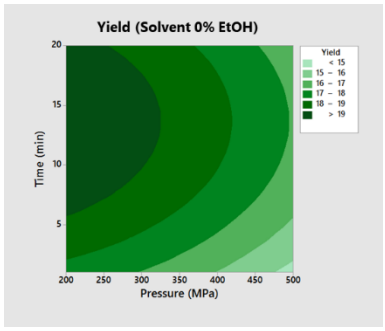


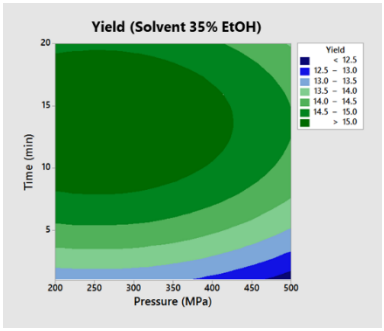
Extraction yield

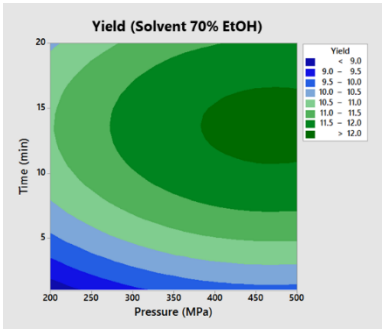




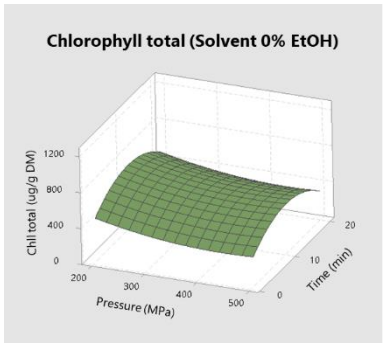


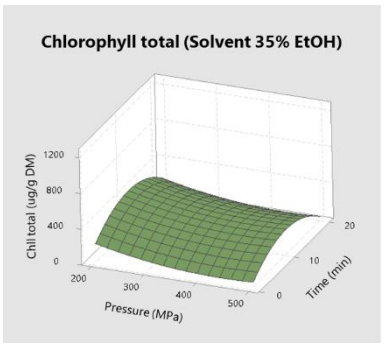


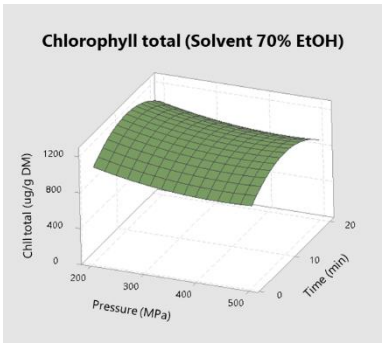


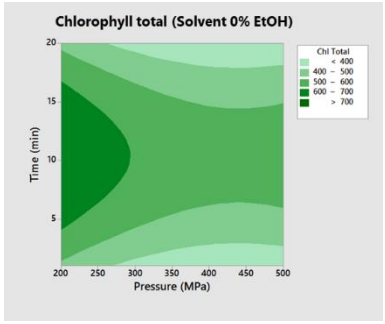


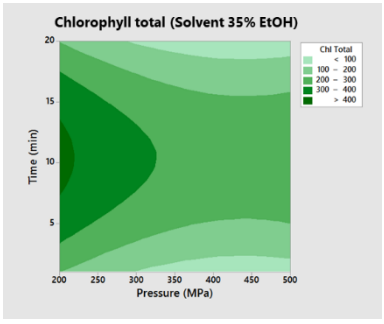
Total chlorophylls

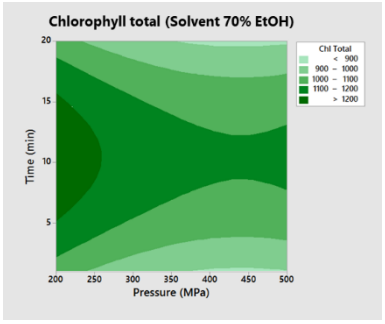












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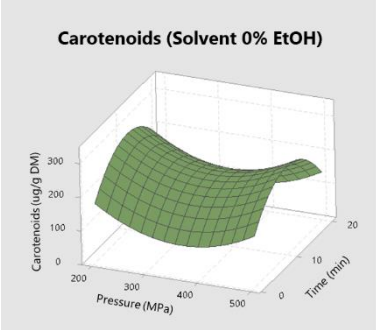
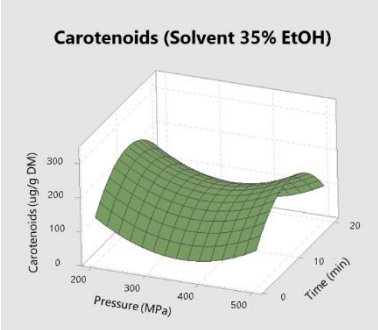
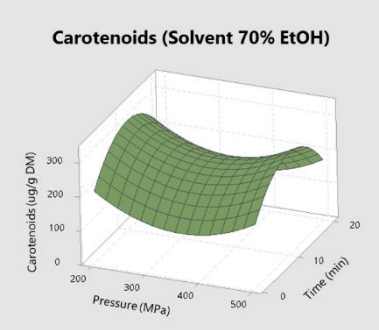
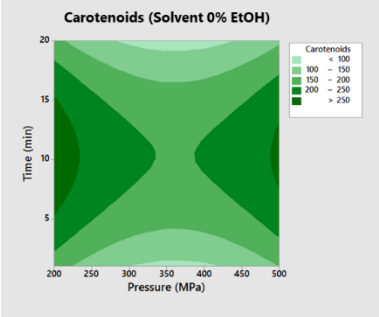
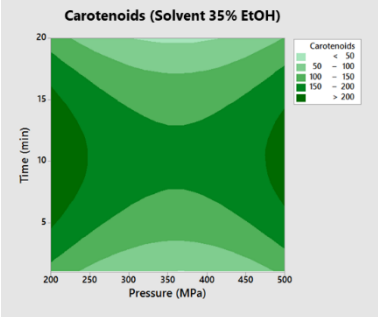
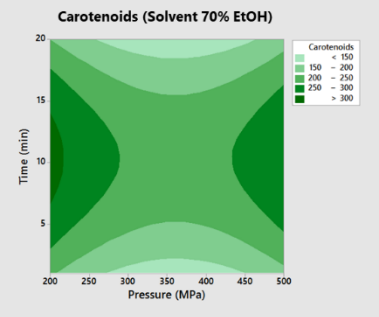






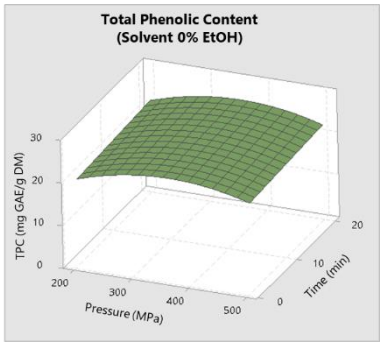


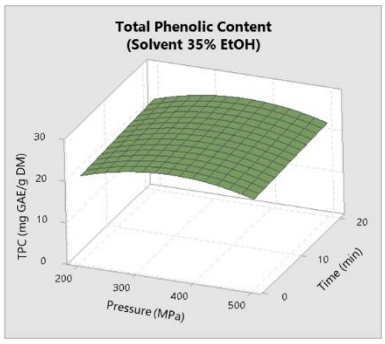


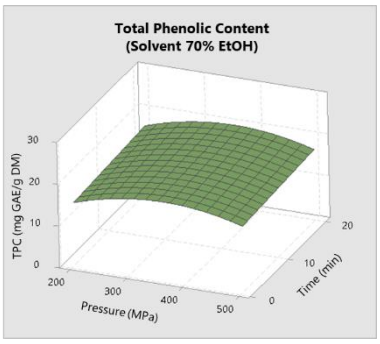


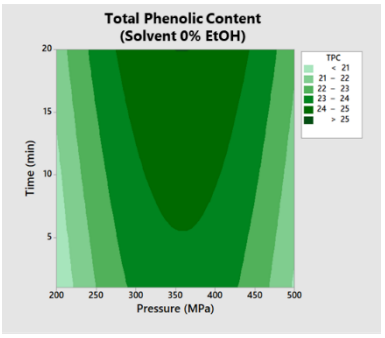
D3 – Response surface graphs for winter savory extraction

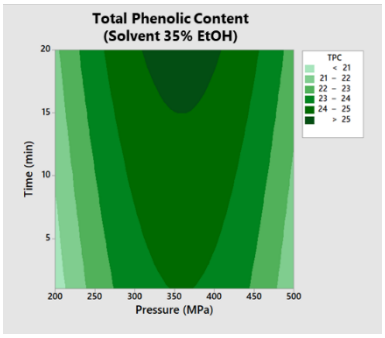
Total phenolic compounds

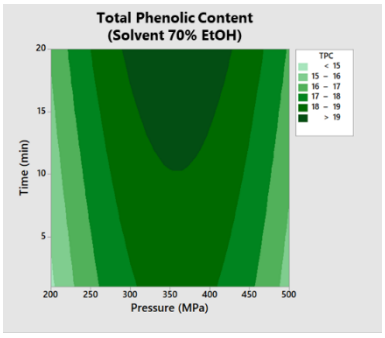




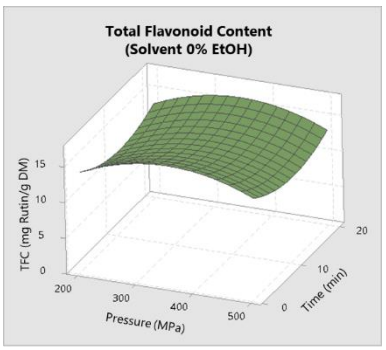


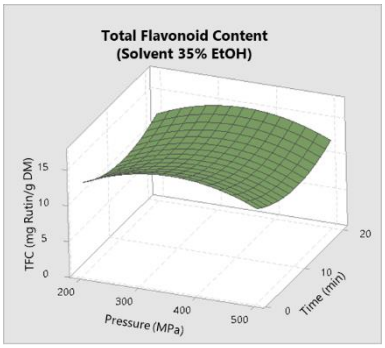


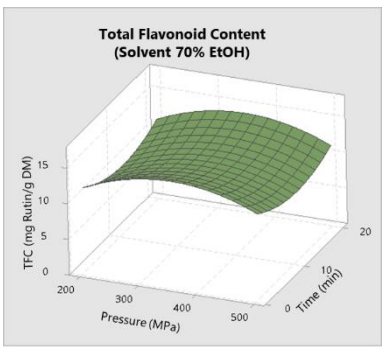


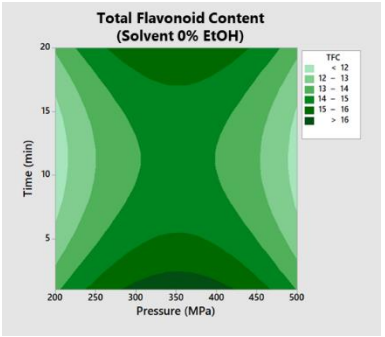


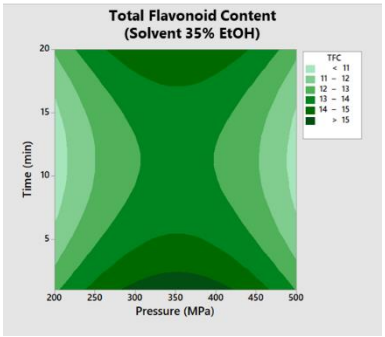
Total flavonoids

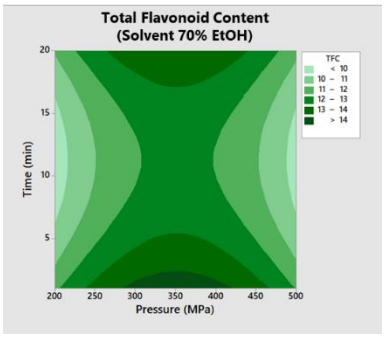




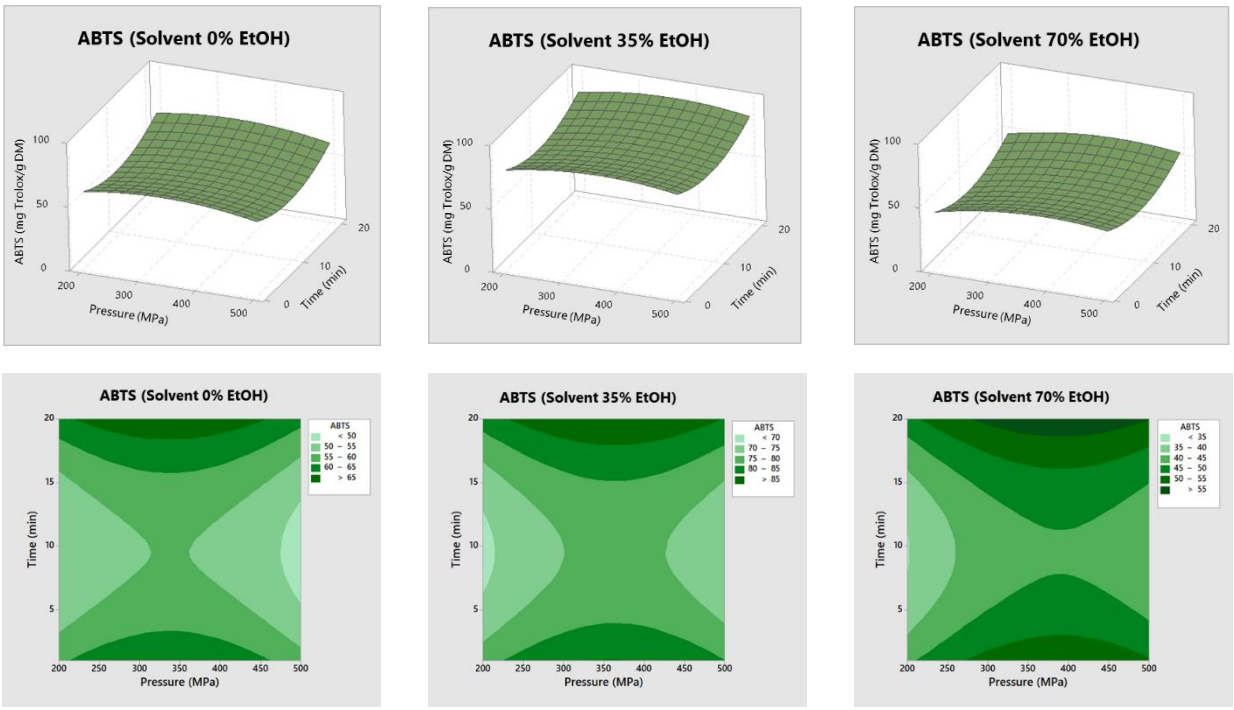




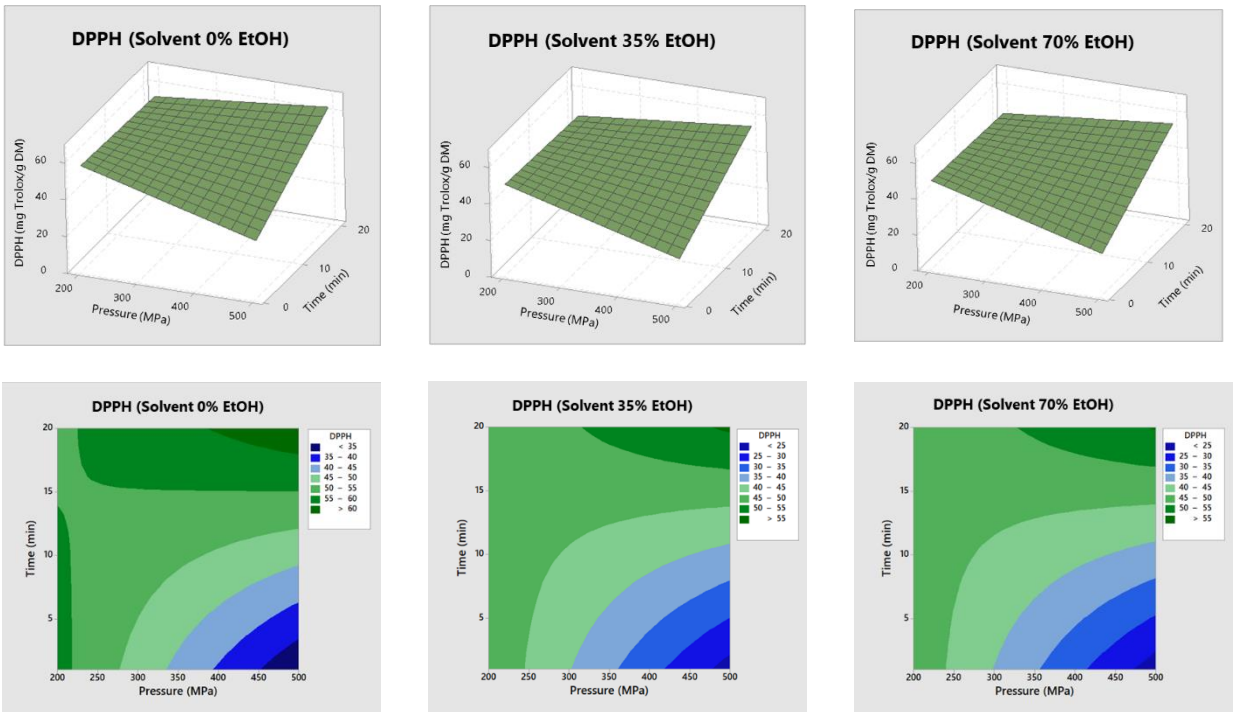




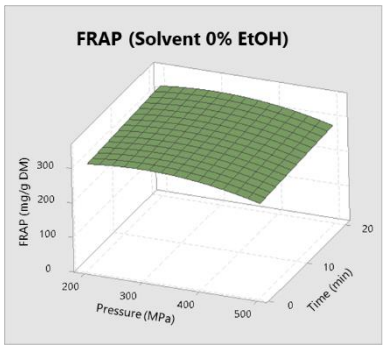
Antioxidant activity by ABTS assay

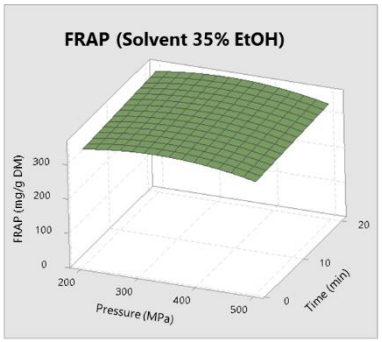


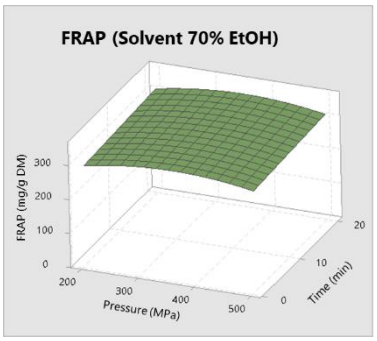
Antioxidant activity by DPPH assay

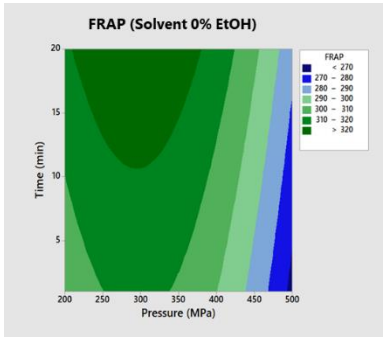


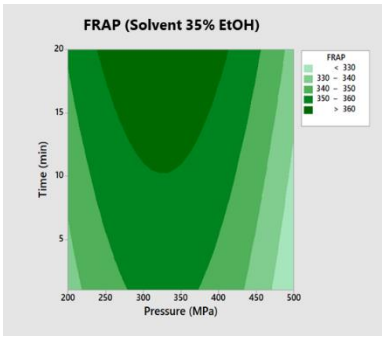
Antioxidant activity by FRAP assay

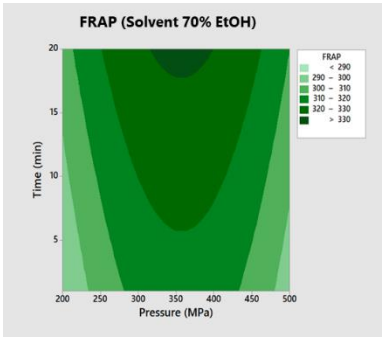




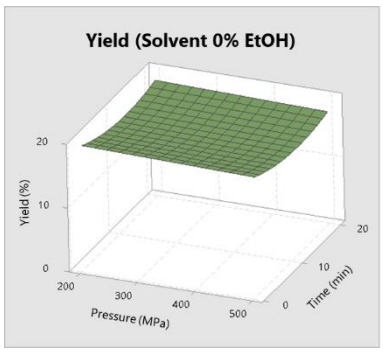


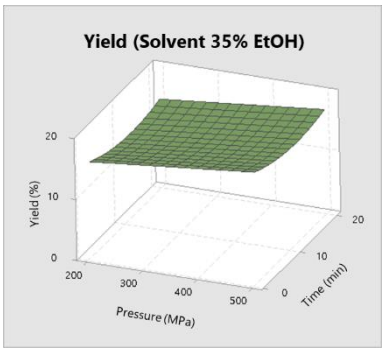


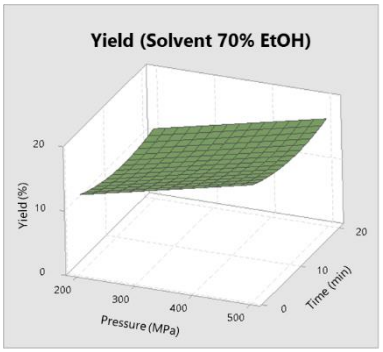


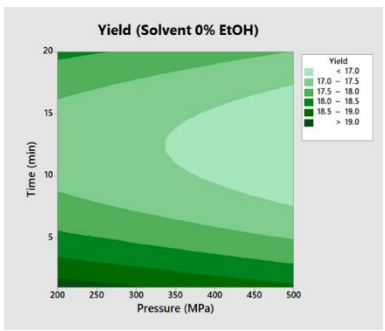


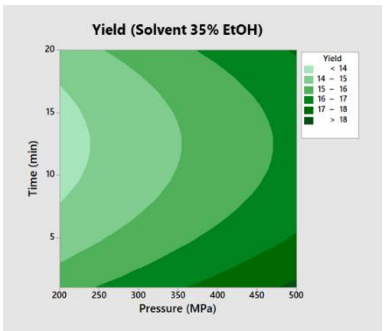
Extraction yield

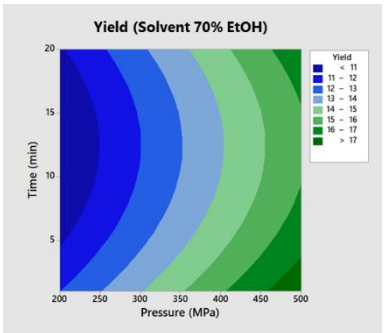




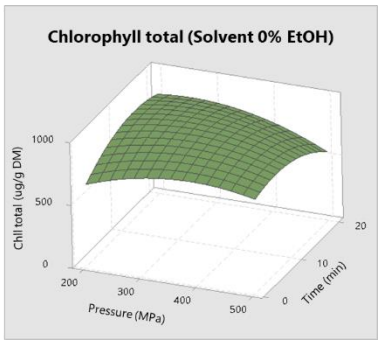


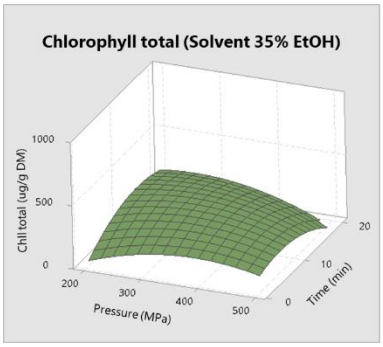


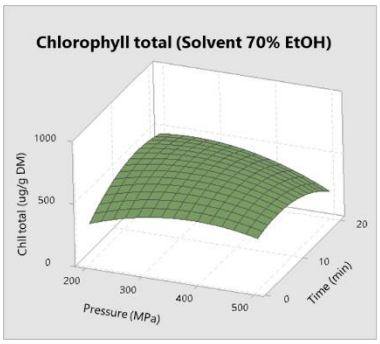


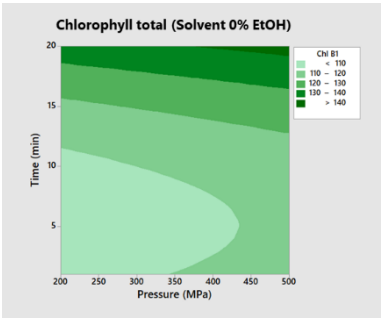


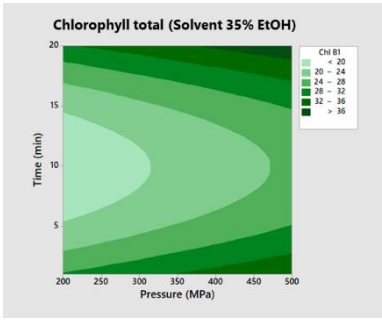
Total chlorophylls

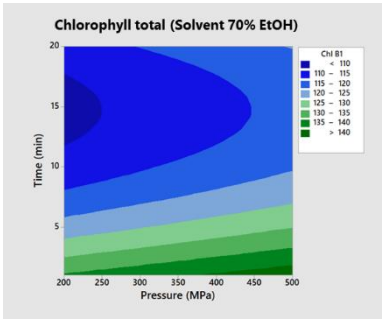




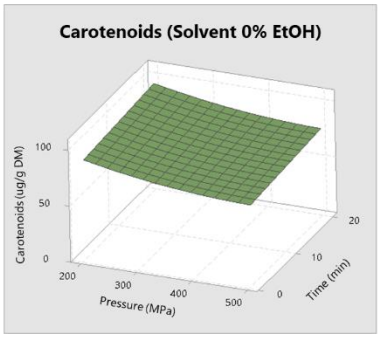


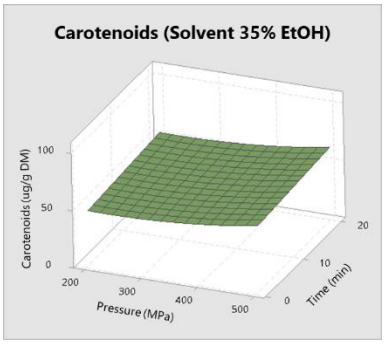


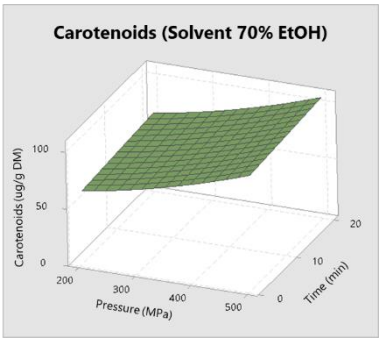


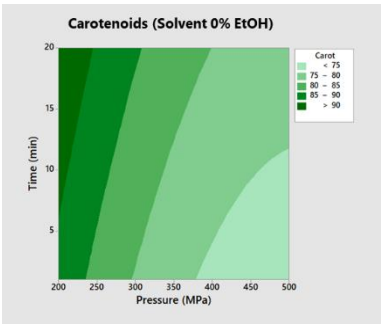


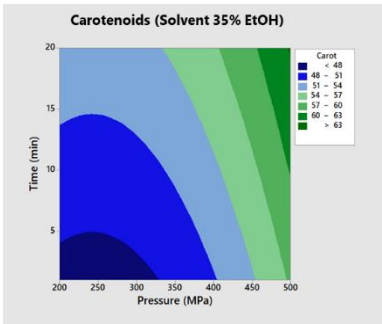
Total carotenoids

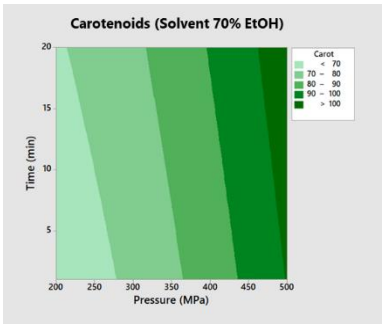












APPENDIX E

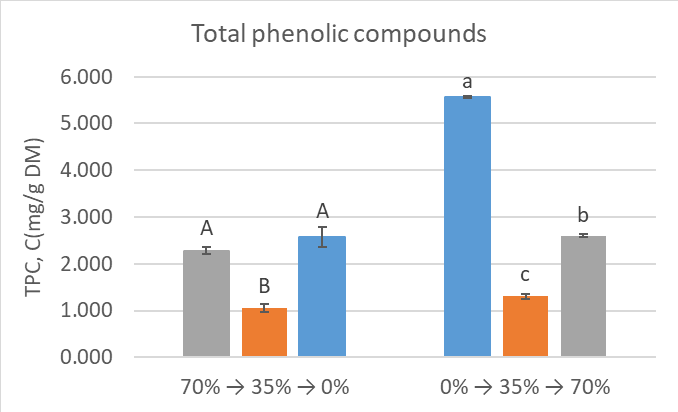
SCREENING TESTS

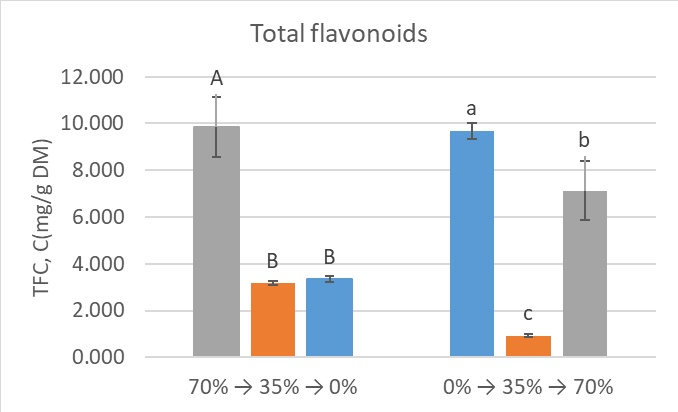
E1 – Stinging nettle

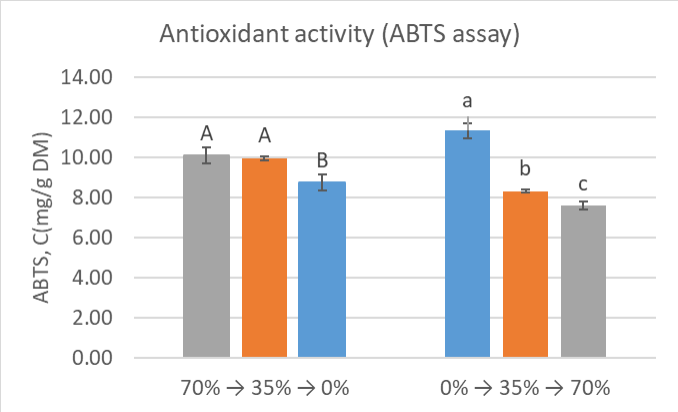
E1.1 – Sequential extraction

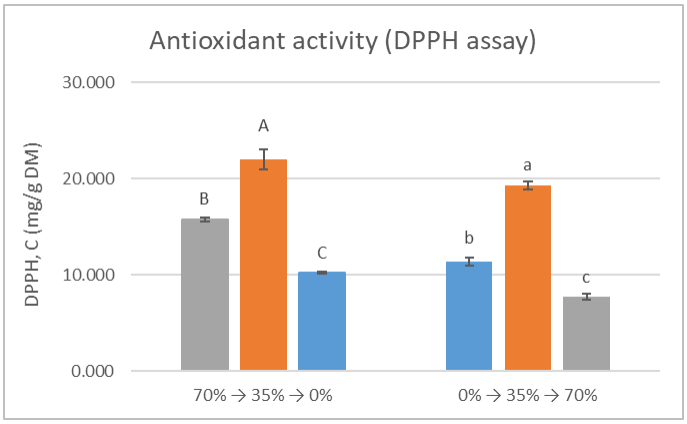
Sequential extraction test aimed to verify if by using the same initial herb in consecutive extractions, using the different solvent mixtures, the different analysed parameters would remain stable. For so, two major tests were performed, (I) initial extraction with 70% ethanol, followed by 35% ethanol, followed by water (70% → 35% → 0%); and (II) initial extraction with water, followed by 35% ethanol, followed by 70% ethanol (0% → 35% → 70%). Different letter indicate significant ($p>0.05$) differences between the values; capital letters indicate the statistical analysis for sequential extraction I, and lowercase letter indicates statistical analysis for sequential extraction II.

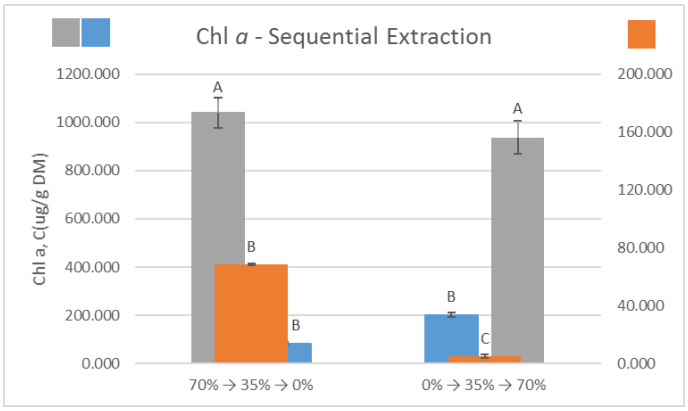
This test allowed to understand how the solvent would influence the concentration of the extracted compounds. For example, for total phenolic compounds, it is clear that an initial extraction using water enables to obtain about twice the concentration of an initial extraction using 70% ethanol; whereas for total chlorophylls it is not important if the extraction begins with water or 70% ethanol, since the majority of these compounds tends to be extracted with non-polar solvents.

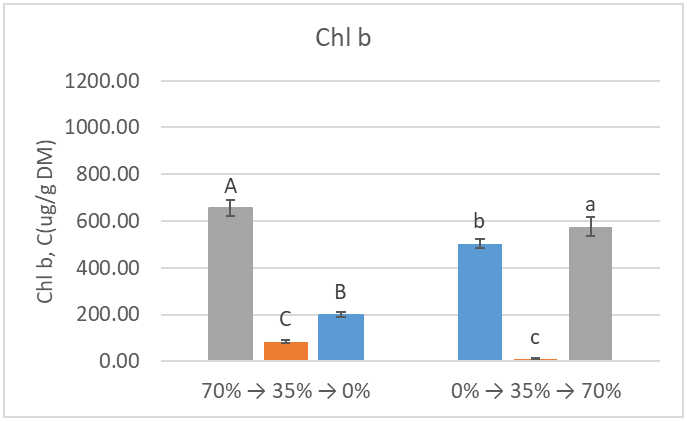


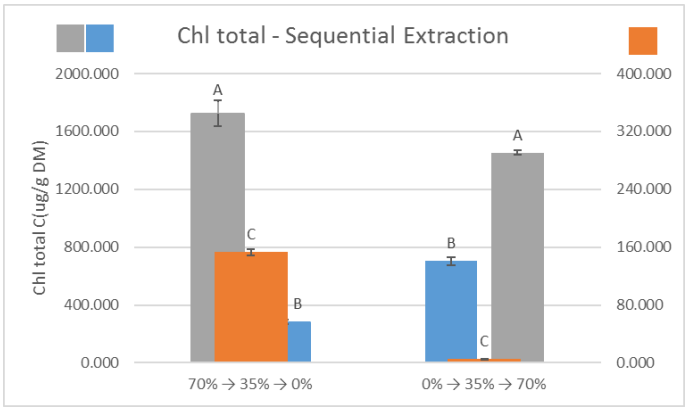


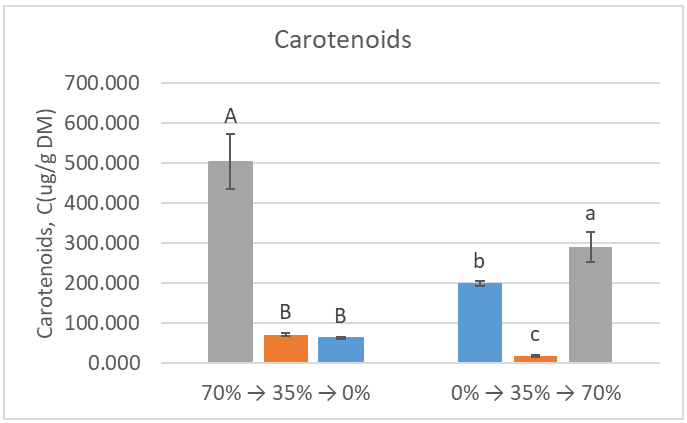


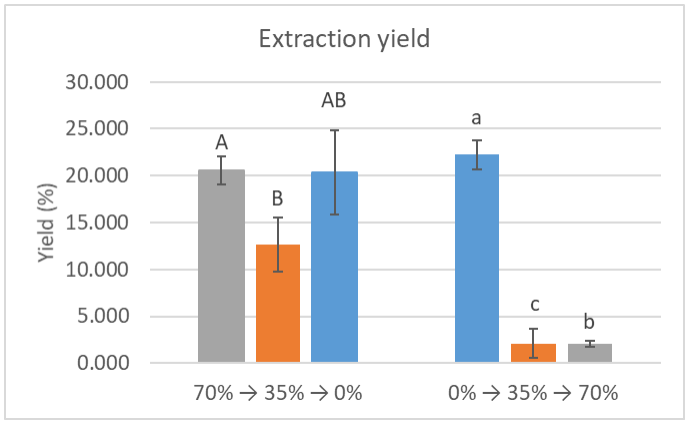



Chl a - Sequential Extraction
Chl a, C(ug/g DM)
1200.000
1000.000
800.000
600.000
400.000
200.000
0.000
200.000
160.000
120.000
80.000
40.000
0.000
A
B
B
B
C
A
70% → 35% → 0%
0% → 35% → 70%


Chl b
Chl b, C(ug/g DM)
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
A
C
B
b
c
a
70% → 35% → 0%
0% → 35% → 70%


Chl total - Sequential Extraction
Chl total C(ug/g DM)
2000.000
1600.000
1200.000
800.000
400.000
0.000
400.000
320.000
240.000
160.000
80.000
0.000
A
C
B
B
C
A
70% → 35% → 0%
0% → 35% → 70%


Carotenoids
Carotenoids, C(ug/g DM)
700.000
600.000
500.000
400.000
300.000
200.000
100.000
0.000
A
B
B
b
c
a
70% → 35% → 0%
0% → 35% → 70%


Extraction yield
Yield (%)
30.000
25.000
20.000
15.000
10.000
5.000
0.000
A
B
AB
a
c
b
70% → 35% → 0%
0% → 35% → 70%

E1.2 – HPLC analysis

HPLC analysis (**Figure E1**) in stinging nettle optimized extracts allowed to perceive if the extracts were rich/poor in total phenolic compounds.

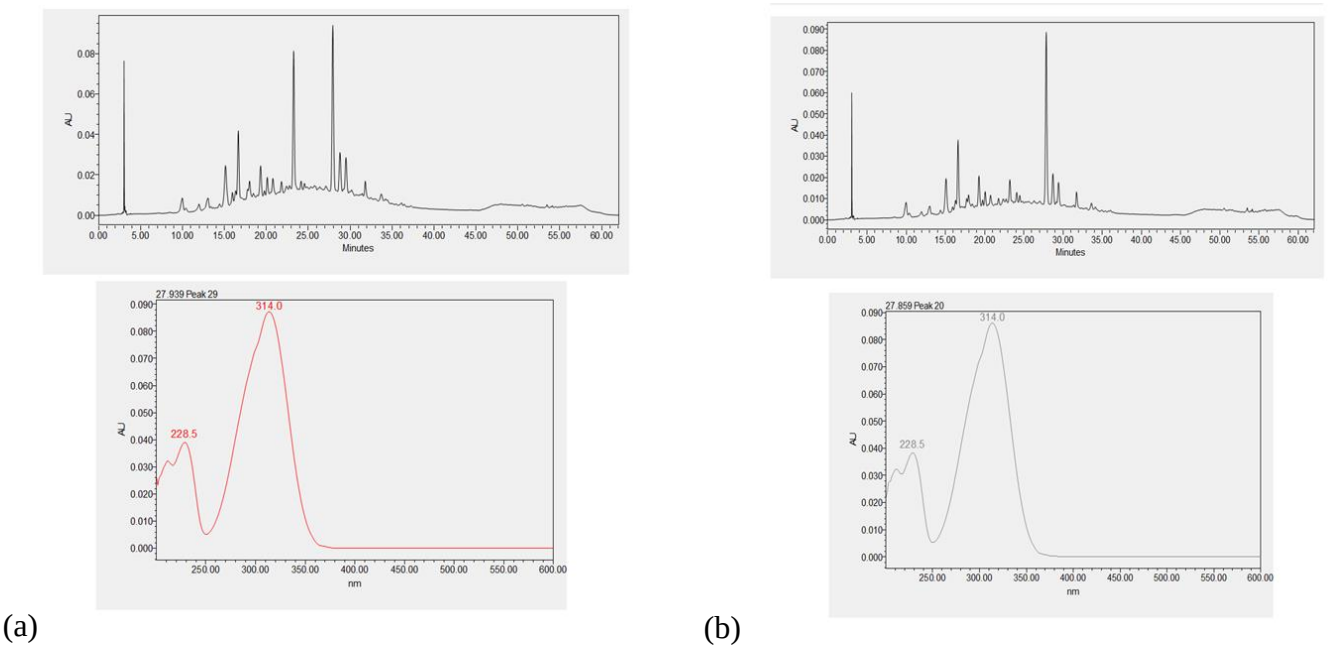


Figure E1. Example of the chromatograms obtained for HPE extracts (a) and control extracts (b). Below the chromatograms is the spectra of the 'unknown' compound, with maximum absorbance at 314.0 nm

This analysis allowed to do a first identification of the major compounds present in the extracts (by comparison to the given libraries), and to understand if there were new compounds still not described for this herb, or not present in the libraries.

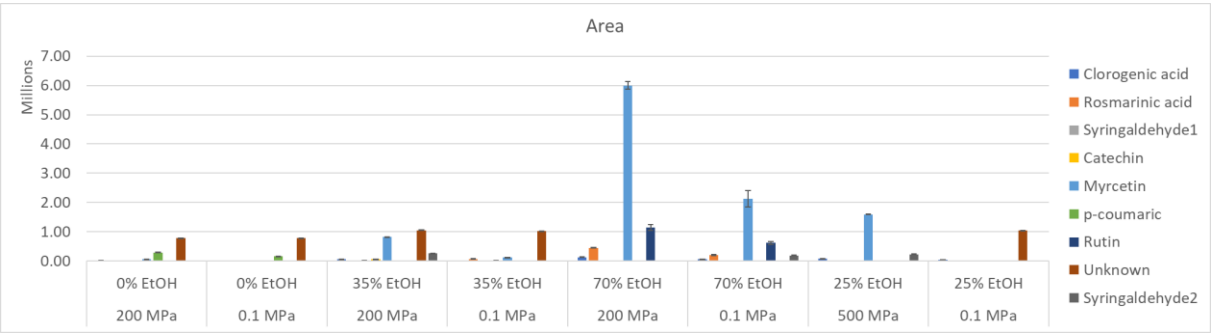


Figure E2. Extracts composition concerning the area of the major compounds found in stinging nettle leaves by HPLC analysis

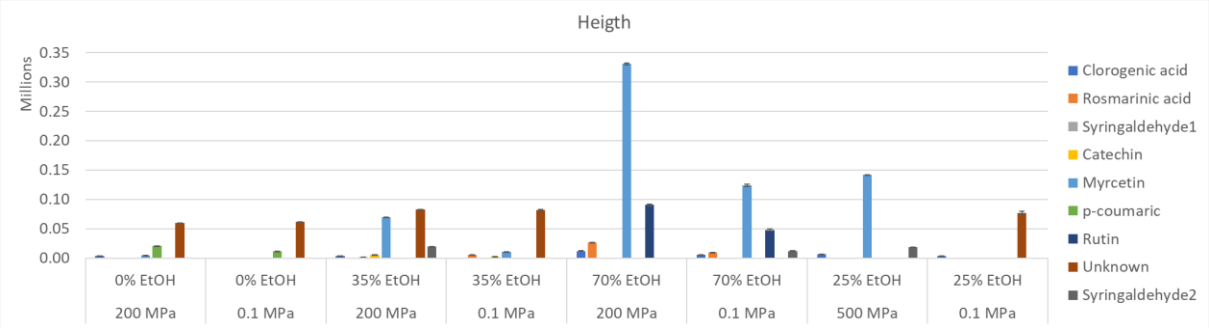


Figure E3. Extracts composition concerning the height of the major compounds found in stinging nettle leaves by HPLC analysis

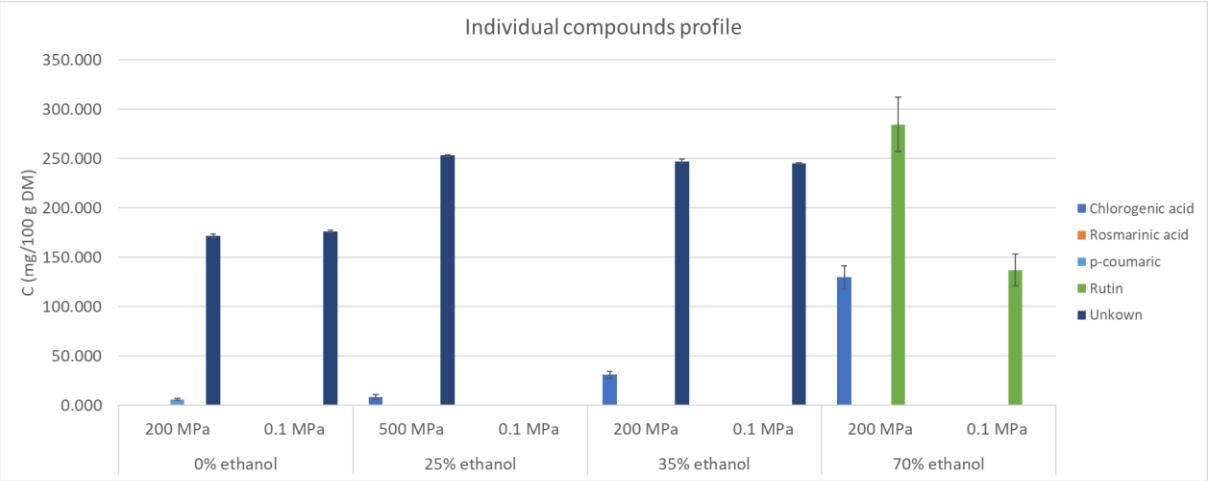


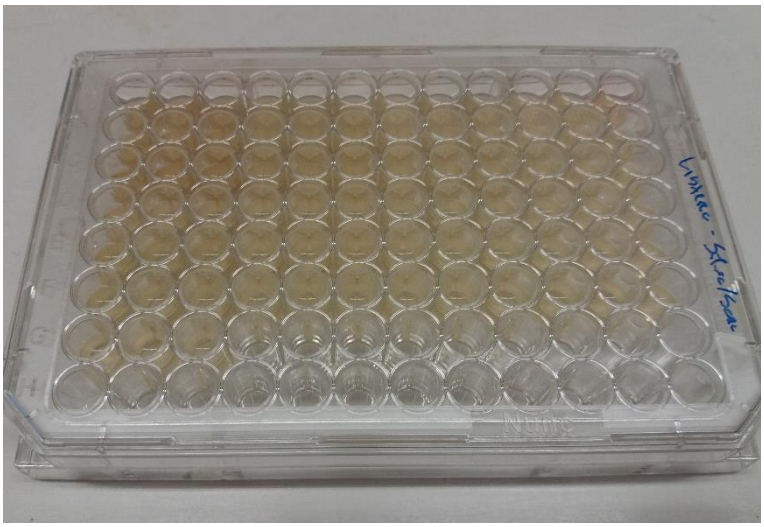
Figure E4. Extracts composition concerning the major compounds found in winter savory leaves by HPLC analysis

The analysis of the **Figures E2, E3**, and **E4** allowed to verify that there was one 'unknown' compound present in all extracts (**Figure E1**), being necessary to perform an LC-MS/MS analysis to identify and quantify it.

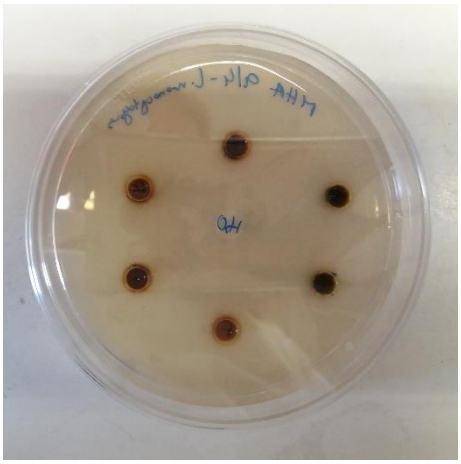
E1.3 – Antimicrobial and antibiofilm activity; potential as prebiotic

Antimicrobial and antibiofilm activities were studied in stinging nettle extracts. Unfortunately, all the microplates used in these tests appear to be contaminated/with high microbial growth and no results on antimicrobial activity could be reported for the tested concentrations (1.0 and 50 mg/mL). Additionally, it was performed a study of formation of an inhibition halo against *Listeria monocytogenes,* but also these analyses seemed to be contaminated//with high microbial growth (**Figure E5**).

(a)



(b)



(c)

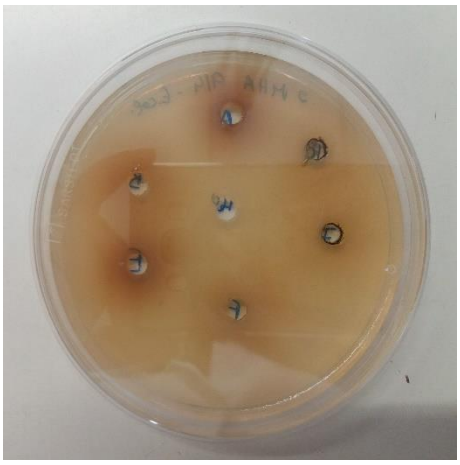


Figure E5. (a) Microplate with *L. monocytogenes* mixed with stinging nettle extracts in order to evaluate their antimicrobial activity. (b) Petri dish with *L. monocytogenes* already with stinging nettle extracts in the wells. (c) Same Petri dish after 24h, clearly contaminated

Nevertheless, the microplates were still used to verify if the extracts had some effect on antibiofilm formation for the microorganisms in study. The **Figures E6-E8** show the major results, demonstrating that the HPE extracts seem to have a higher biofilm formation inhibition than the controls, especially against *Staphylococcus aureus* (25.28 ± 2.98 to 28.69 ± 5.15% for N200/10.6/35 against 14.82 ± 2.38 to 24.02 ± 2.06 for N0.1/10.6/35).

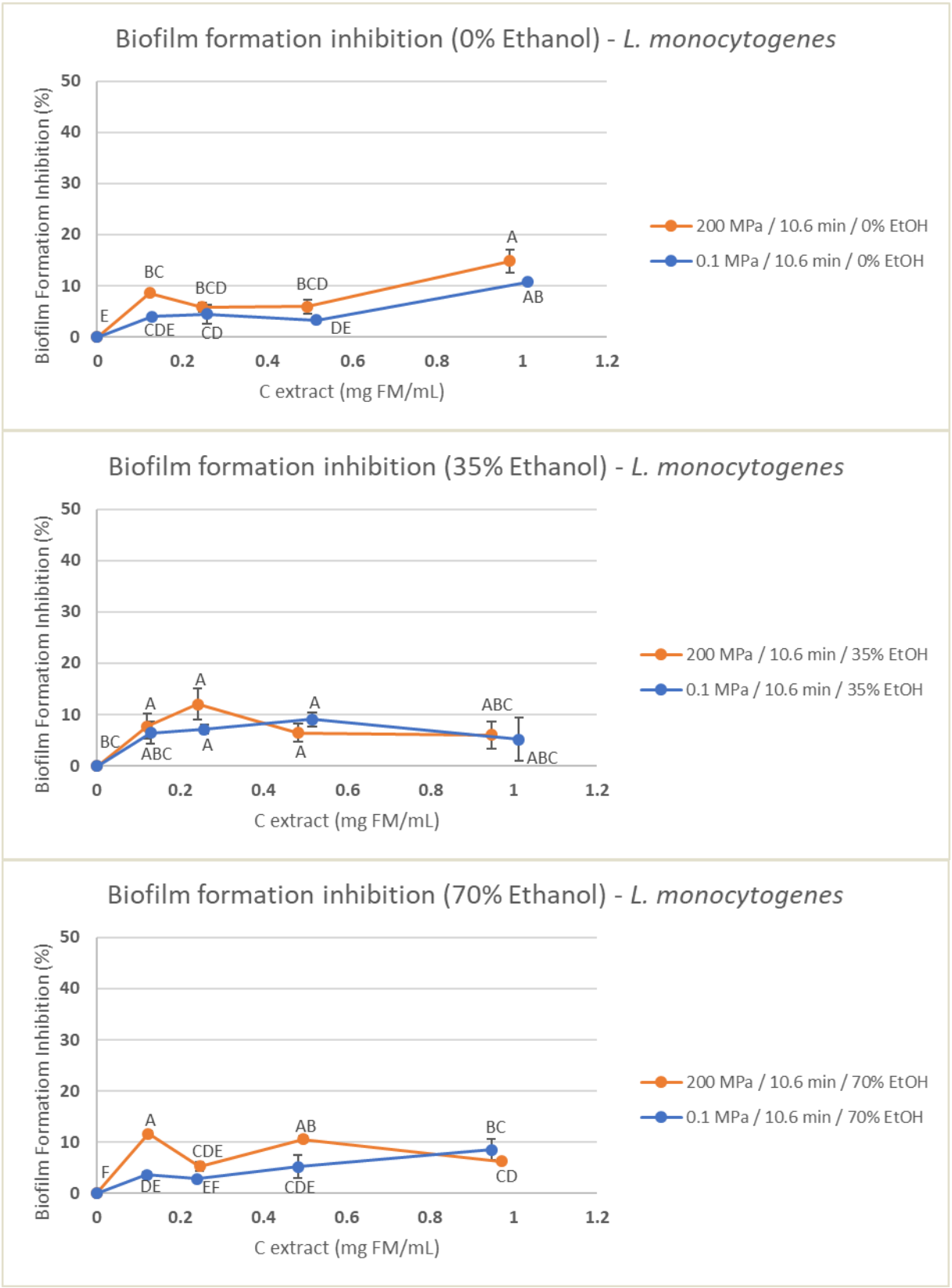


Figure E6. Results for antibiofilm activity of stinging nettle extracts (aqueous, 35% ethanol, and 70% ethanol) against *L. monocytogenes*

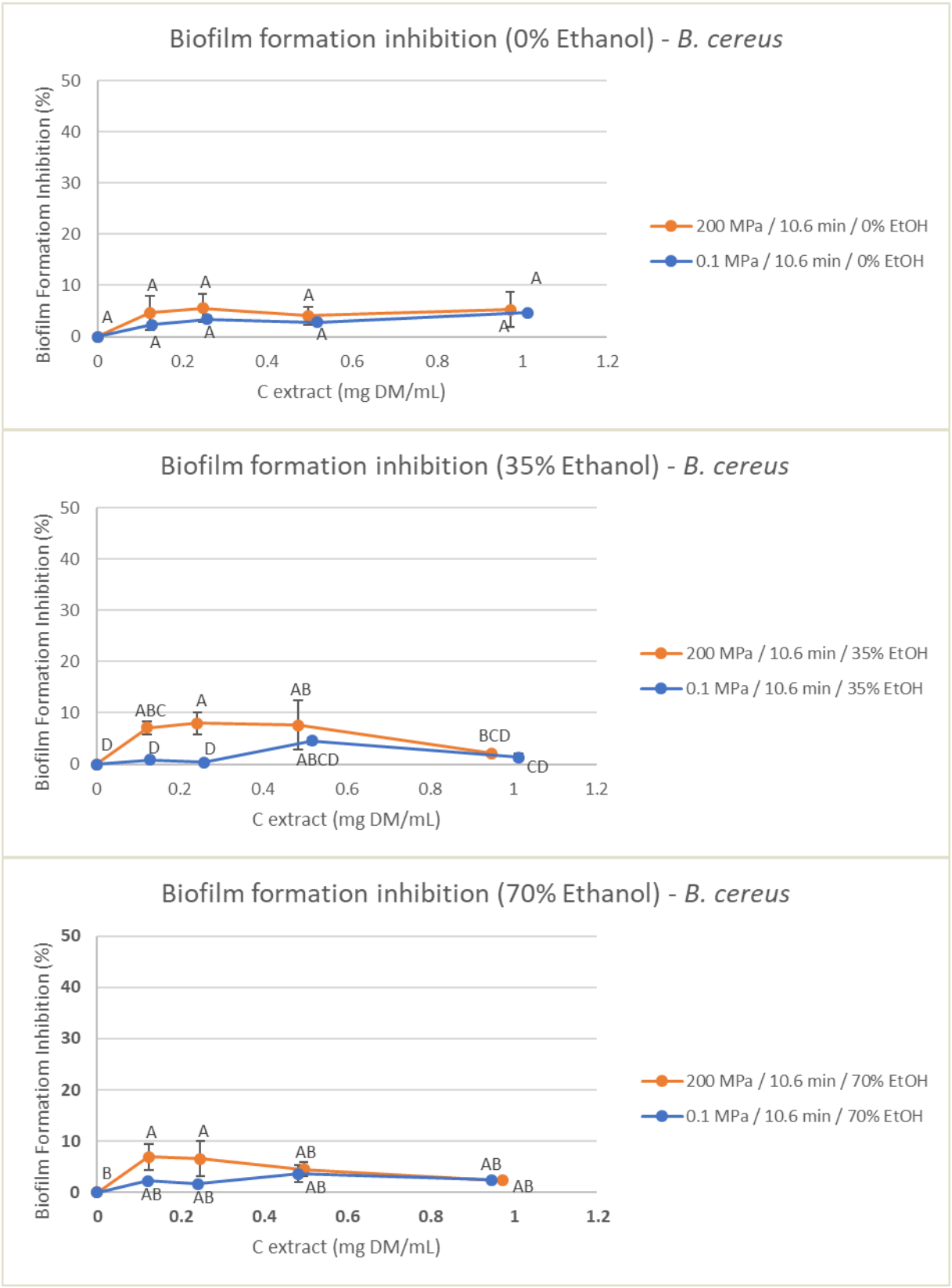


Figure E7. Results for antibiofilm activity of stinging nettle extracts (aqueous, 35% ethanol, and 70% ethanol) against *B. cereus*

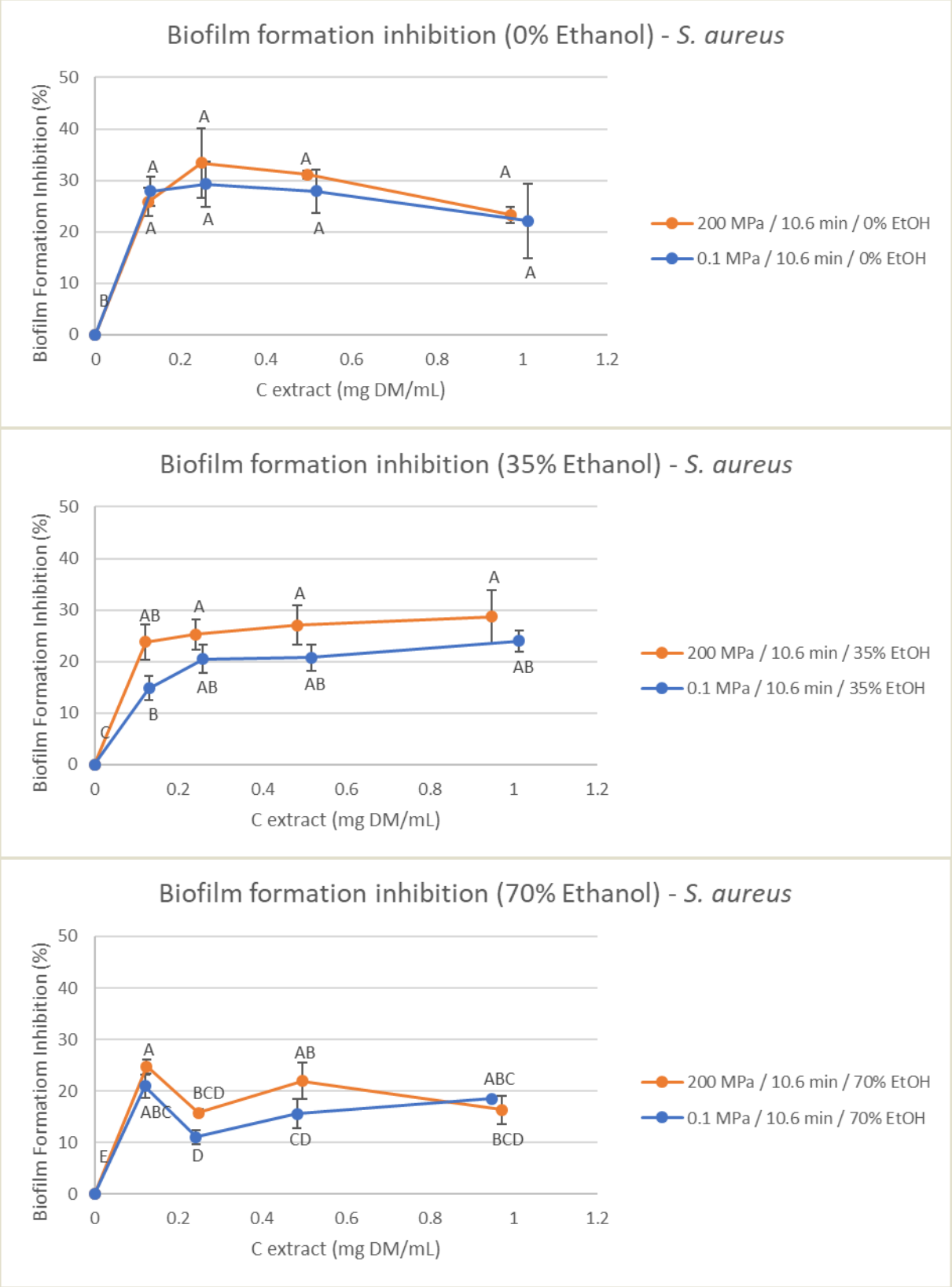


Figure E8. Results for antibiofilm activity of stinging nettle extracts (aqueous, 35% ethanol, and 70% ethanol) against *Staphylococcus aureus*

Since stinging nettle extracts (2.0 mg/mL) seemed to be improving microbial growth instead of inhibiting it, a study concerning the extracts potential as prebiotic was performed on the growth of *Lactobacillus acidophilus* (**Figure E9**).

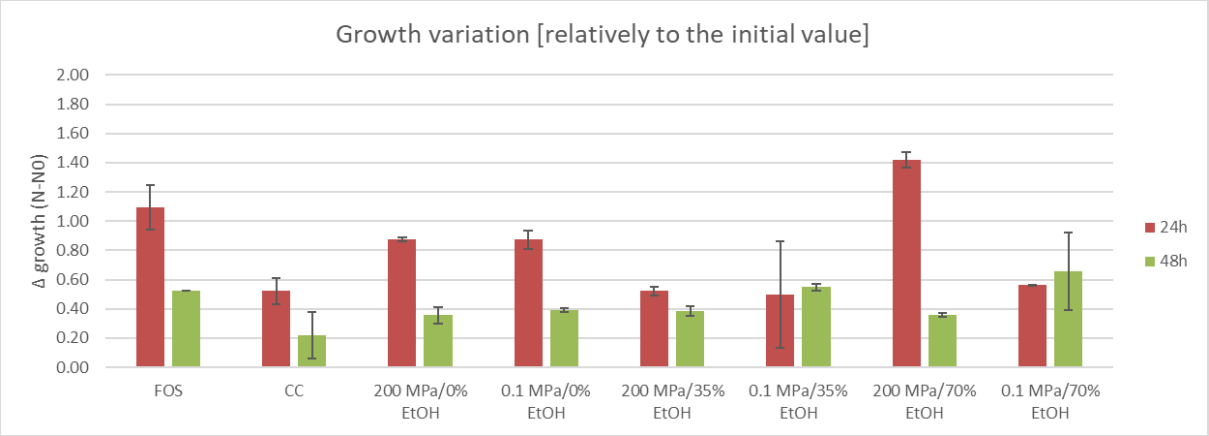


Figure E9. Growth variation (compared to the initial value) of *L. acidophilus* in the presence of stinging nettle extracts. 'FOS' is the positive control (mixture of fructooligosaccharide and inoculum) and 'CC' is the negative control (mixture of culture medium and inoculum).

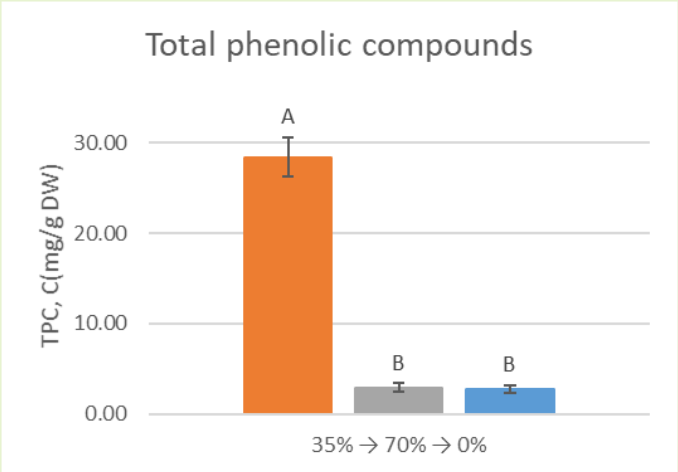
The only extract with some potential as pre-biotic was the N200/10.6/70 (in the **Figure E9** identified as 200 MPa/70% EtOH), since after 24 h of incubation it presented values of growth variation of *L. acidophilus* similar to the FOS (positive control, fructooligosaccharide).

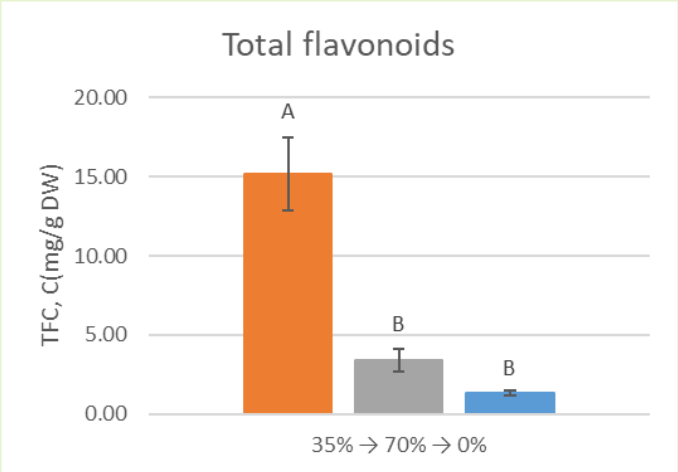
E2 – Winter savory

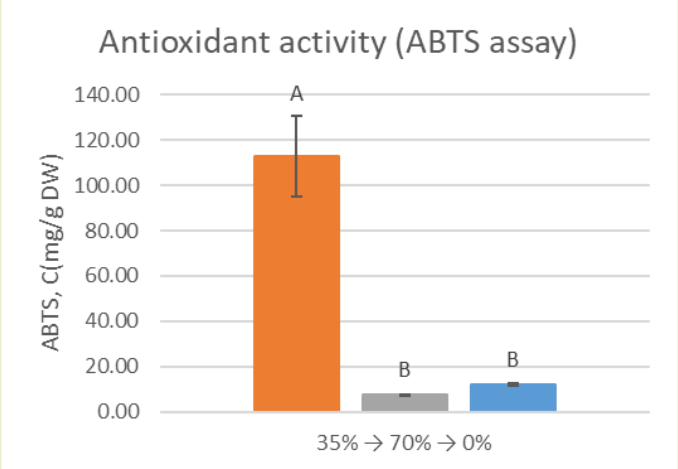
E2.1 – Sequential extraction

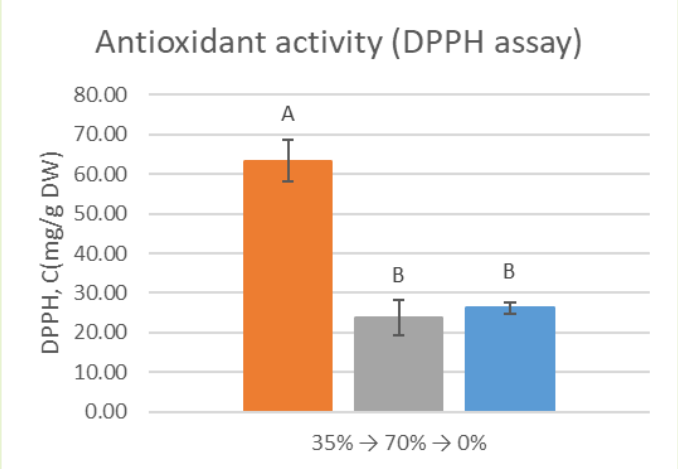
Sequential extraction test aimed to verify if by using the same initial herb in consecutive extractions, using the different solvent mixtures, the different analysed parameters would remain stable. For so, a test was performed: initial extraction with 35% ethanol, followed by 70% ethanol, followed by water (for the final extract to be aqueous). Different letters indicate significant ($p>0.05$) differences between the values; capital letters indicate the statistical analysis for sequential extraction I, and lowercase letter indicates statistical analysis for sequential extraction II.

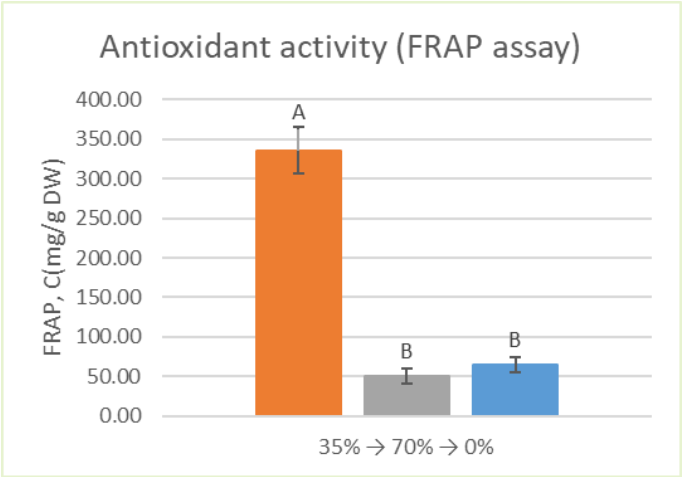
This test allowed to understand that 35% ethanol would be the most suited solvent for extraction of total phenolic compounds, total flavonoids, and higher antioxidant activity obtained by the three studied methods; whereas, once more, 70% ethanol would be the most suitable solvent for pigments extraction.

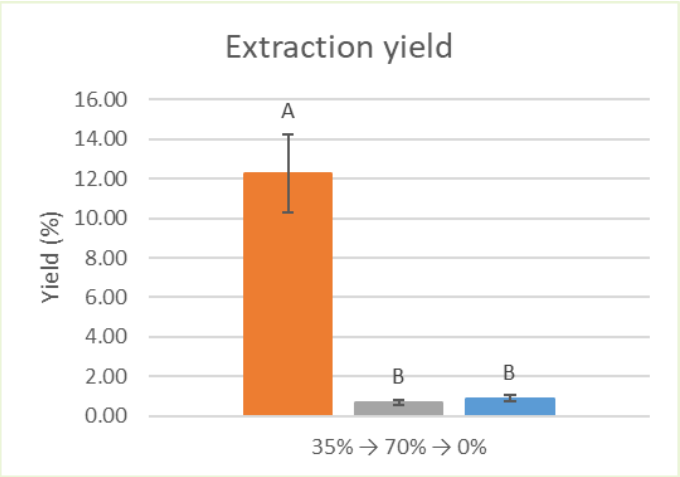


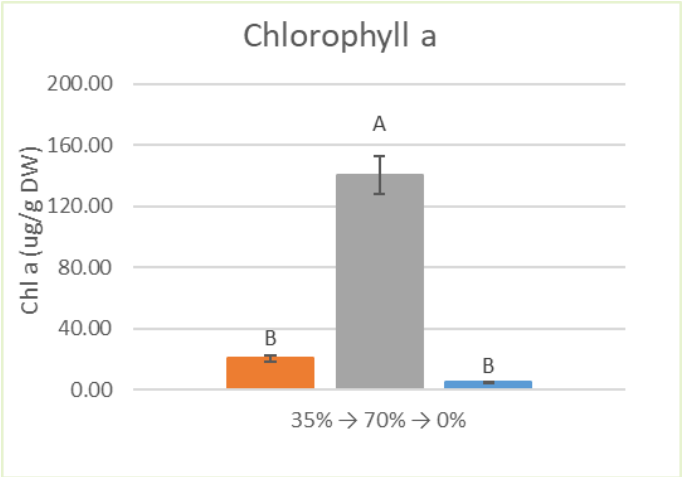


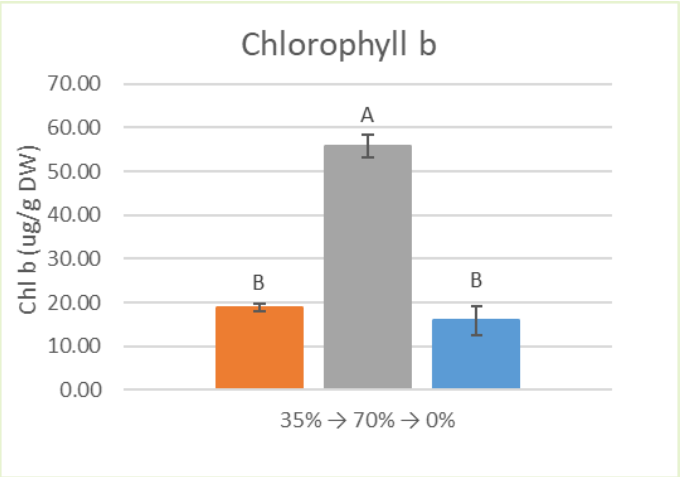


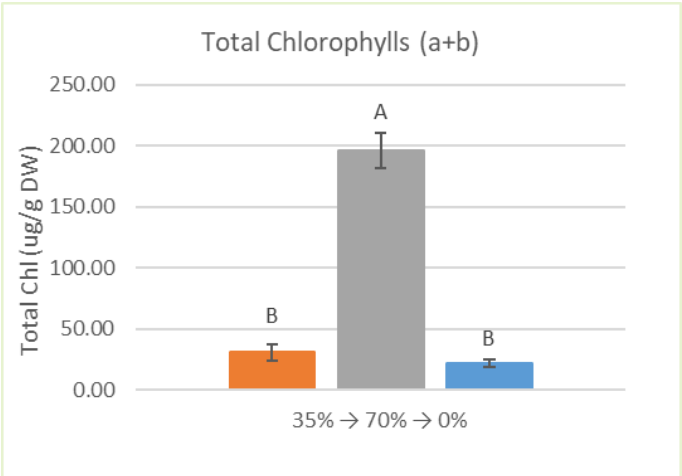


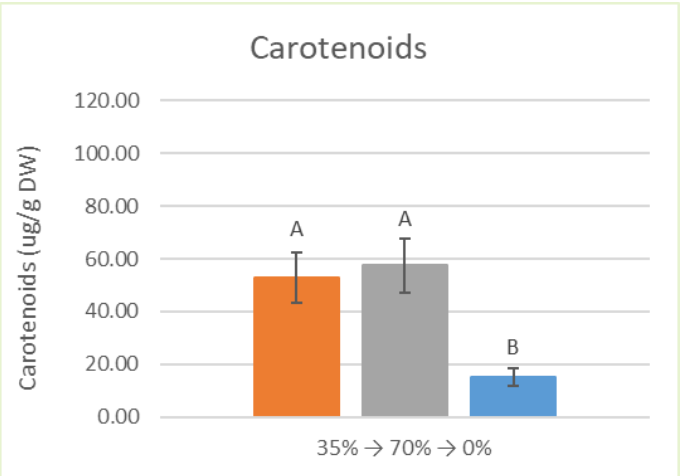

Antioxidant activity (FRAP assay)
FRAP, C(mg/g DW)
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
A
B
B
35% → 70% → 0%


Extraction yield
Yield (%)
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
A
B
B
35% → 70% → 0%


Chlorophyll a
Chl a (ug/g DW)
200.00
160.00
120.00
80.00
40.00
0.00
B
A
B
35% → 70% → 0%

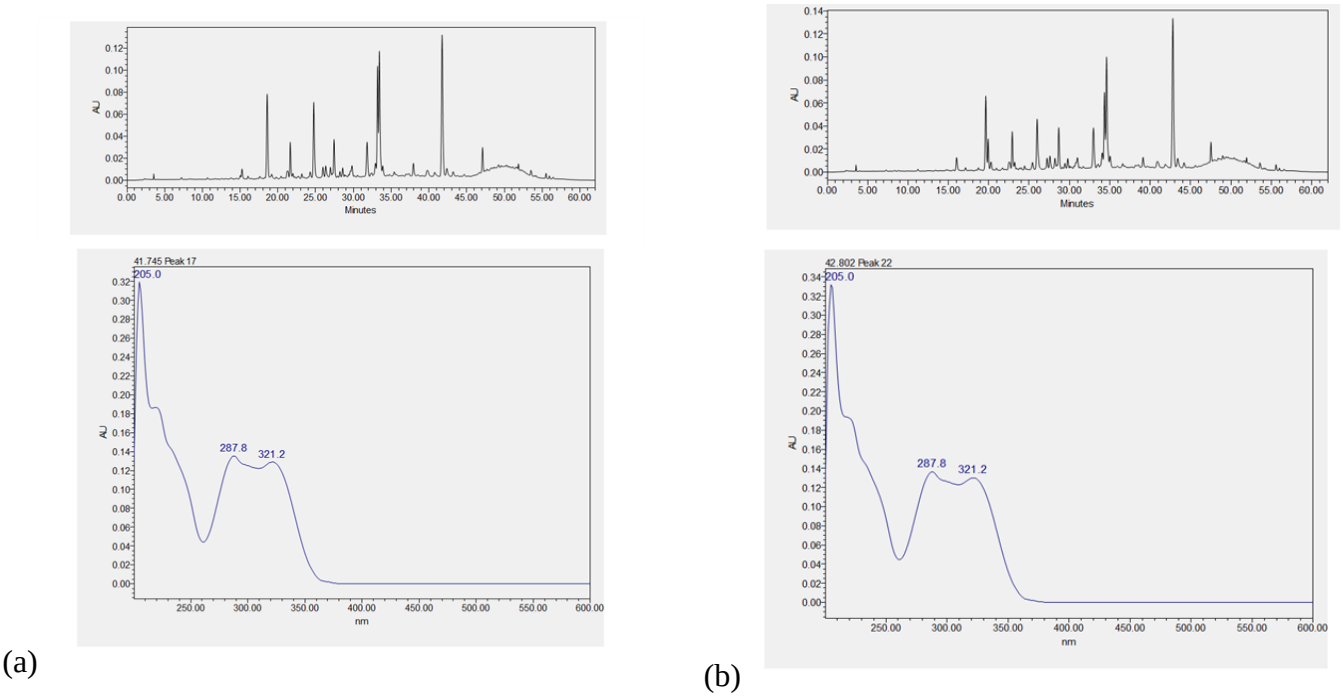

Chlorophyll b
Chl b (ug/g DW)
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
B
A
B
35% → 70% → 0%


Total Chlorophylls (a+b)
Total Chl (ug/g DW)
250.00
200.00
150.00
100.00
50.00
0.00
B
A
B
35% → 70% → 0%


Carotenoids
Carotenoids (ug/g DW)
120.00
100.00
80.00
60.00
40.00
20.00
0.00
A
A
B
35% → 70% → 0%

E2.2 – HPLC analysis

HPLC analysis (**Figure E10**) in winter savory optimized extracts allowed to perceive if the extracts were rich/poor in total phenolic compounds.



(a) (b)

Figure E10. Example of the chromatograms obtained for HPE extracts (a) and control extracts (b). Below the chromatograms is the spectra of the 'unknown' compound, with maximum absorbance at 287.8 and 321.2 nm

This analysis allowed to do a first identification of the major compounds present in the extracts (by comparison to the given libraries, and quantification by standard curves – **Appendix B**, **section B3**), and to understand if there were new compounds still not described for this herb, or not present in the libraries.

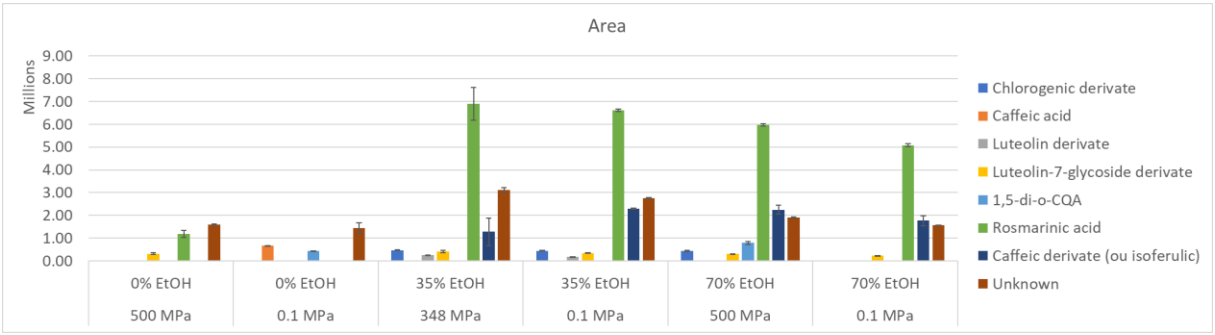


Figure E11. Extracts composition concerning the area of the major compounds found in winter savory leaves by HPLC analysis

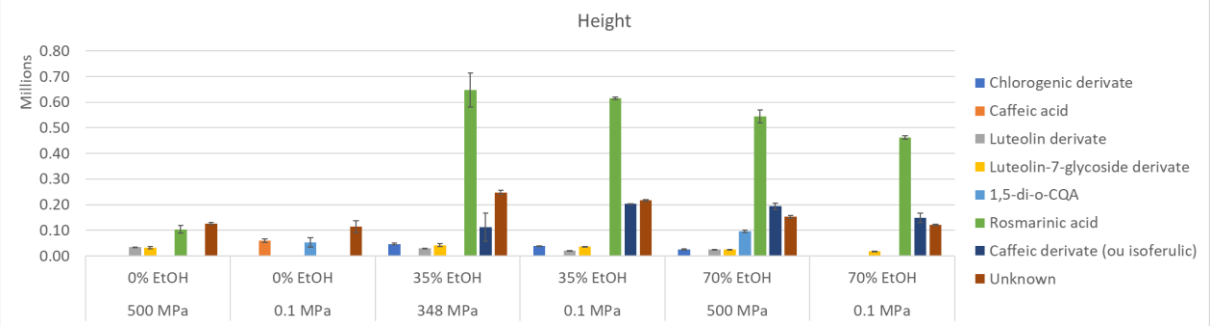


Figure E12. Extracts composition concerning the height of the major compounds found in winter savory leaves by HPLC analysis

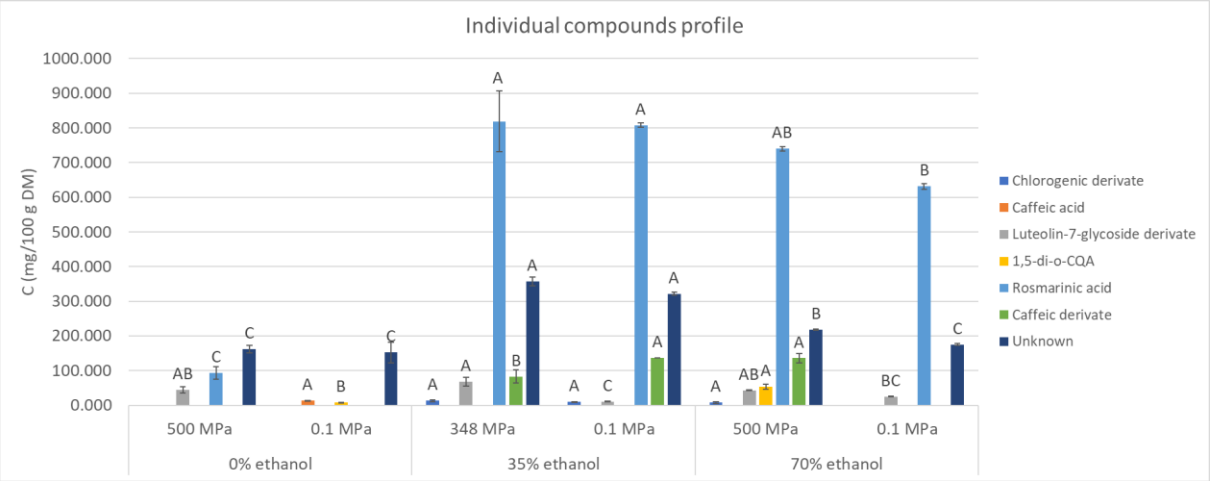


Figure E13. Extracts composition concerning the major compounds found in winter savory leaves by HPLC analysis

Similarly to what happened with stinging nettle extracts, also for winter savory extracts there was verified that there was one 'unknown' compound present in all extracts (**Figure E10)**, being necessary to perform an LC-MS/MS analysis to identify and quantify it.

APPENDIX F

SELECTION OF SECOND HERBAL SAMPLE

In order to select the second herbal sample for the present thesis, a screening study was performed through the literature on the number of published articles and concerning the extraction process (**Table F1**). After choosing the herbs with fewer studies on extraction process and content of bioactive compounds, a screening on their biological activities' potential was performed (**Table F2**).

Table F1. List of possible samples. Marked in green are the samples with fewer published articles, with focus on the extraction processes.

Possible sample				
Portuguese common name	**English common name**	**Scientific name**	**Number of papers**	**Number of papers concerning extraction process**
Absinto	Absinthe/ Wormwood	*Artemisia absinthium*	930	40
Acorus	Acorus	*Acorus gramineus*	2167	36
Agastache	Agastache	*Agastache foeniculum*	818	1
Agerato	-	*Achillea ageratum*	43	1
Arruda	Rue	*Ruta graveolens*	145	38
Artemisia	Sagebrush	*Artemisia annua*	3414	217
Balsamita	Balsamite	*Tanacetum balsamita*	5	3
Calêndula	Calendula	*Calendula officinalis*	2594	102
Coentros	Coriander	*Coriandrum sativum*	4031	141
Cravinas	Clove pink	*Dianthus caryophyllus*	113	27
Cravo-túnico	Marigold	*Tagetes sp.*	2825	10
Erva-camaleão	-	*Houttuynia cordata var. 'Chameleon'*	1625	88 (var. chameleon - 0)
Erva-cidreira	Lemongrass / Bee balm	*Melissa officinalis*	1047	137
Erva-das-azeitonas	Calamints	*Calamintha baetica*	18	2
Erva-do-caril	Curry plant	*Helichrysum italicum*	777	14
Estêva	gum rockrose	*Cistus ladanifer*	448	24
Falso-boldo	-	*Plectranthus Barbatus*	109	6
Feno-de-cheiro	Sweet vernal grass	*Anthoxanthum odoratum*	754	15
Hipericão-do-Gerês	Tutsan	*Hypericum androsaemum*	148	3
Hortelã-de-cabra	Canary Islands-balm	*Cedronella canariensis*	7	0
Hortelã-mourisca	Water mint	*Mentha aquatica*	246	11
Hortelã-pimenta	White peppermint	*Mentha x piperita officinalis*	108	8

Hortelã-vietnamita	Vietnamese coriander	*Polygonum odoratum*	47	3
Hortelã-vulgar	Spearmint	*Mentha spicata*	1192	82
Jasmim	Jasmine	*Jasminum officinale*	67	1
Limonete	Lemon Beebrush	*Aloysia triphylla*	193	23
Lírio-florentina	Iris	*Iris x germanica var. 'Florentina'*	235	11
Macela-camomila	Roman Chamomile	*Chamaemelum nobile*	198	18
Manjericão	Basil	*Ocimum basilicum*	2935	221
Manjericão-roxo	Basil	*Ocimum basilicum 'purpurea'*	24	2
Milfólio	Yarrow	*Achillea millefolium*	1597	82
Mirra-bastarda	Myrrh	*Plectranthus sp.*	47	3
Orégão-dourado	Oregano	*Origanum vulgare*	2392	179
Orégão-vulgar	Oregano	*Origanum vulgare*	2392	179
Poêjo	Pennyroyal	*Mentha pulegium*	447	32
Rapazinhos	Blackcurrant Sage	*Salvia microphylla*	43	2
Rosmaninho-africano	Wild rosemary	*Eriocephalus africanus*	17	0
Rosmaninho-maior	Lavender	*Lavandula pedunculata*	40	2
Rúcula	Wall Rocket	*Diplotaxis muralis*	75	1
Salsa	Parsley	*Petroselinum crispum*	1682	98
Salva	Common sage	*Salvia officinalis*	3267	392
Salva-icterina	Common sage	*Salvia officinalis Icterina*	2	0
Salva-tricolor	Common sage	*Salvia officinalis 'Tricolor'*	10	1
Santolina	Cotton lavender	*Santolina chamaecyparissus*	106	4
Santolina verde	Green santolina	*Santolina virens*	6	0
Segurelha	Winter savory	*Satureja montana*	283	42
Tomilho-dos-gatos	Cat thyme	*Teucrium marum*	33	2
Tomilho-limão	Lemon thyme	*Thymus x citriodorus*	18	2
Tomilho-vulgar	Thyme	*Thymus vulgaris*	3255	251
Tormentelo	Headed savory	*Thymus caespititius*	45	7

Table F2. List of the possible samples concerning the most known biological activities for each one

English common name	Scientific name	Antiox.	Anti-inflam.	Antimicr.	Analgesic	Healing	Digestive	Laxative	Cosmetic	Respiratory system	Calming	Anti-cholesterol	Rheumatism	Diuretic
-	***Achillea ageratum***				x	x	x						x	
Balsamite/ Costmary	***Tanacetum balsamita***			x		x	x	x						
Calamints/ Calamintha	***Calamintha baetica***	x		x			x		x	x				
Canary Islands balm	***Cedronella canariensis***						x			x	x	x		
Vietnamese coriander	***Polygonum odoratum***						x						x	x
Wild rosemary	***Eriocephalus africanus***	x	x	x			x		x	x			x	x
Lavender	***Lavandula pedunculata***	x	x	x		x	x				x			x
Common sage	***Salvia officinalis***	x		x			x				x			
Winter savory	***Satureja montana***			x	x	x	x		x	x				x
Green santolina	***Santolina virens***		x	x		x	x		x	x	x			
Cat thyme	***Teucrium marum***						x			x				x
Lemon thyme	***Thymus x citriodorus***		x	x						x			x	
Headed savory	***Thymus caespititius***			x										