Andreia Raquel Simões Cunha
Licenciada em Engenharia Química e Bioquímica

Valorization of plant materials by Supercritical fluid technology

Dissertação para obtenção do Grau de Mestre em Engenharia Química e Bioquímica

Orientador: Prof.ª Doutora Susana Barreiros, Professora Associada com Agregação, FCT-UNL

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Presidente: Prof. Doutor Mário Fernando José Eusébio, Professor Auxiliar da FCT-UNL
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FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Resumo

Este trabalho teve como objetivo a produção de extractos das plantas *Pterospartum tridentatum* (Carqueja) *e Waltheria indica* utilizando dióxido de carbono supercrítico ($scCO_2$), um solvente considerado verde, aqui utilizado puro, ou com etanol como co-solvente. Os óleos essenciais destas plantas têm importância económica devido aos seus compostos com inúmeras propriedades medicinais. Para comparação, realizaram-se ensaios de extracção com Soxhlet/etanol e infusão/água. Os rendimentos de extracção mais elevados foram obtidos nas extracções com infusão/água - 22,5% para a Carqueja e 25,0% para a W. *indica* – seguidos de Soxhlet/etanol - 21,5% para a Carqueja e 22,4% para a W. *indica*. No caso das extracções com $scCO_2$, obtiveram-se maiores rendimentos de extracção a 500 bar do que a 350 bar à temperatura de 40 ºC – 1.28% e 0.64%, respectivamente. Estes valores subiram quando, à mesma temperatura e a 300 bar, se adicionou ao CO_2 etanol como co-solvente – máximo de 4.36% para Carqueja e 14% p/p de etanol, e máximo de 3.13% para a *Waltheria indica* e 10% p/p de etanol. Realizaram-se ainda ensaios de extracção com Soxhlet/etanol e infusão/água de Carqueja e W. *indica* previamente extraídas com $scCO_2$, com ou sem co-solvente etanol. A tendência verificada foi para um aumento dos rendimentos de extracção quando se utilizou Soxhlet/etanol, sugerindo que o $scCO_2$ conseguiu retirar de ambas as plantas compostos que o método Soxhlet/etanol não permitia extraír. Já no caso da infusão/água, a tendência verificada foi para uma diminuição dos rendimentos de extracção relativamente aos valores obtidos com o material original, sugerindo que o $scCO_2$ conseguiu retirar de ambas as plantas alguns compostos que por infusão/água também são extraídos. Tendo em conta que, tanto a Carqueja, como a *W. indica*, apresentam os polifenóis como compostos maioritários, os extratos foram analisados com o objetivo de quantificar o teor em compostos fenólicos (TPC) e a capacidade antioxidante, esta última através do índice EC_{50} (concentração que induz metade do efeito máximo). Os melhores valores para a Carqueja foram 64, 67 e 118 mg de equivalentes de ácido gálico por g de planta e para extractos obtidos com $scCO_2$/etanol, Soxhlet/etanol e infusão/água, respectivamente, e para a *Waltheria indica* 10, 77 e 91 mg de equivalentes de ácido gálico por g de planta para extractos obtidos com $scCO_2$/etanol, Soxhlet/etanol e infusão/água, respectivamente. Os valores de EC_{50} obtidos foram sempre muito elevados (desfavoráveis) para os extractos de ambas as plantas obtidos com $scCO_2$/etanol, e foram de cerca de 1 e 0.4 g de extracto por g de DPPH (radical utilizado neste teste) para os extractos de Carqueja obtidos com Soxhlet/etanol e infusão/água, respectivamente, 2 e 0.3 g de extracto por g de DPPH para os extractos de *Waltheria indica* obtidos com Soxhlet/etanol e infusão/água, respectivamente. Numa caracterização preliminar dos extractos de Carqueja, realizada por cromatografia em camada fina (TLC), já tinha sido evidenciada a presença de compostos fenólicos nos extractos obtidos com Soxhlet/etanol, bem como ácidos gordos, triglicéridos e terpenos nos extractos obtidos com $scCO_2$, Soxhlet/etanol e Soxhlet/*n*-hexano. Este trabalho pode ser considerado uma primeira

abordagem à utilização da tecnologia supercrítica para obter extractos de Carqueja e de *W. indica*.

Palavras-chave: Extração supercrítica; Dióxido de carbono supercrítico; *Pterospartum tridentatum*; *Waltheria Indica*; Fenólicos; Antioxidantes.

Abstract

The aim of this work was to obtain extracts from two plants, namely *Pterospartum tridentatum* (Carqueja) and *Waltheria indica*, using supercritical carbon dioxide ($scCO_2$), a green solvent. $ScCO_2$ was used pure or with added co-solvent ethanol. The essential oils from these plants have economic value due to their content in compounds with medicinal properties. For comparison, extraction assays were performed using Soxhlet/ethanol and infusion/water. The highest yields of extraction were obtained when using infusion/water - 22,5% for Carqueja and 25,0% for W. *indica* – followed by Soxhlet/ethanol - 21,5% for Carqueja and 22,4% for W. *indica*. In the case of extractions with $scCO_2$, higher yields of extraction were obtained at 500 bar than 350 bar at a temperature of 40 ºC – 1.28% and 0.64%, respectively. These values went up when at the same temperature and 300 bar, ethanol was added to CO_2 as co-solvent – maximum of 4.36% for Carqueja and 14 wt.% ethanol, and maximum of 3.13% for *Waltheria indica* and 10 wt.% of ethanol. Extraction assays were also carried out by Soxhlet/ethanol and infusion/water from Carqueja and W. *indica* previously extracted with $scCO_2$, with or without co-solvent ethanol. The trend observed was an increase in yields of extraction when Soxhlet/ethanol was used, suggesting that $scCO_2$ was able to remove from both plants compounds that the Soxhlet/ethanol method cannot extract. In the case of infusion/water, the trend observed was a decrease in yields of extraction relative to the values obtained with the original plant material, suggesting that $scCO_2$ was able to remove from both plants some compounds that are also extracted by the infusion/water method. Taking into consideration that polyphenols are the major class of compounds present in Carqueja and in *W. indica*, the extracts obtained were analyzed with a view to quantifying total phenolic compounds (TPC) and antioxidant capacity, the latter through values of EC_{50} (half maximum effective concentration). The best results for Carqueja were 64, 67 and 118 mg of gallic acid equivalents per g of plant and for extracts obtained with $scCO_2$/ethanol, Soxhlet/ethanol and infusion/water, respectively, and for *Waltheria indica* 10, 77 and 91 mg of gallic acid equivalents per g of plant and for extracts obtained with $scCO_2$/ethanol, Soxhlet/ethanol and infusion/water, respectively. The EC_{50} values were always very high (unfavorable) for extracts from both plants obtained by $scCO_2$/ethanol extraction, and were of ca. 1 and 0.4 g of extract per g of DPPH (radical used in this assay) for Carqueja extracts obtained by Soxhlet/ethanol and infusion/water, respectively, and 2 and 0.3 g of extract per g of DPPH for *Waltheria indica* extracts obtained by Soxhlet/ethanol and infusion/water, respectively. A preliminary characterization of Carqueja extracts performed by thin layer chromatography (TLC) had already evidenced the presence of phenolic compounds in extracts obtained by Soxhlet/ethanol, as well as fatty acids, triglycerides and terpenes in extracts obtained by $scCO_2$, Soxhlet/ethanol and Soxhlet/*n*-hexane extraction. This work can be considered a first approach to the use of supercritical technology to obtain extracts from Carqueja and *W. indica*.

Keywords: Supercritical extraction; Supercritical Carbon Dioxide; *Pterospartum tridentatum*; *Waltheria Indica*; Phenolic content; Antioxidants.

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List of abbreviations and symbols

µL – Microliter

µm – Micrometer

λ – Wavelength

λ_{max} – Maximum wavelength

Abs – Absorbance

ASE – Accelerated solvent extraction

BPR – Back Pressure Regulator

°C – Celsius

Carq – Carqueja

cm – Centimeter

CO_2 – Carbon dioxide

DPPH – 2,2-diphenyl-1-picrylhydrazyl

FC – Folin-Ciocalteau reagent

g – Gram

GAE – Gallic acid equivalence

h – Hour

HPLC –High Performance Liquid Chromatography

K – Kelvin

Kg – Kilogram

L – Litre

m – Meter

m^3 – Cubic meter

min – Minute

mL – Milliliter

mm – Millimeter

MPa – Mega Pascal

P – Pressure

p_c – Critical pressure

P. *tridentatum* - *Pterospartum tridentatum (L.) Willk*

PFE – Pressurized fluid extraction

PHSE – Pressurized hot solvent extraction

PLE – Pressurized liquid extraction

REF – Literature references

RSA – Radical scavenging activity

ROS - Reactive oxygen species

s – Seconds

$scCO_2$ – Supercritical Carbon Dioxide

SCF – Supercritical fluid

SFE – Supercritical fluid extraction

SSE – Subcritical solvent extraction

S&L – Stems and leaves

T - Temperature

T_c – Critical temperature

TCA – Trichloroacetic acid solution

TLC – Thin Layer Chromatography

TPC – Total phenolic compounds

W. *indica* – *Waltheria indica*

WHO - World Health Organization

wt. – Weight

CHAPTER 1: State of the art

1.1 Extraction Processes

Both plants and plant-based products are an important source of nutrients, whether it is for health care or nutrition**.** The World Health Organization (WHO) estimates that around 70% to 80% of the world population uses plants and plant products for their benefit [1]. Over the last few years, the international demand for essential oils obtained from plant raw materials has increased and has led to the development of new extraction processes, aiming to improve the quality of the extracts obtained, as well as lowering operational costs [2].

Several methods of extraction have been utilized. Traditional approaches include, for instance, infusion, maceration, Soxhlet and steam distillation [2]. These extraction methods are often simple and practical, but they have drawbacks, such as employing hazardous processing solvents like hexane, benzene, pentane and various chlorinated solvents, which need to be removed from the extracts and recovered. This requires additional steps and can generate large amounts of organic waste, raising environmental risk issues [3]. Also many extraction methods employ heat, which can lead to damaged aroma compounds, decreased molar mass and degradation of heat-labile molecules [1,3]. Aditionally, yields of extraction are often low.

In the 1990s, green chemistry approaches began to be applied to extraction processes, namely by replacing many conventional solvents with less environmentally harmful alternatives. Among greener technologies, there are methods such as microwave extraction, ultrasound-assisted extraction, pressurized fluid extraction (PFE), and supercritical fluid extraction (SFE) [2,3]. In **Table 1** there is a summary of the advantages and disadvantages of the greener extraction methods**.** The pressurized fluid extraction (PFE) technique uses solvents that are taken closely to the supercritical region. High temperatures (50-200°C) combined with high pressures (35-200 bar) allow for a fast extraction (5-10 mins) and a high yield, because while the solubility of target compounds and solvent diffusion rate in the sample increase, the viscosity and the surface tension of the solvent decrease. PFE has an advantage over traditional techniques since it needs a smaller amount of solvent, is fast, and the sample is not exposed to light or oxygen. PFE is also known as subcritical solvent extraction (SSE), pressurized hot solvent extraction (PHSE), or accelerated solvent extraction (ASE) [2].

In the last few decades, one of the technologies that improved the extraction of natural products was supercritical fluid extraction [4]. This extraction method stands out for the quality of the extracts obtained. Of the aforementioned technologies, only supercritical fluid extraction (SFE) grants both an alternative solvent approach and reduced processing energy inputs. Consequently, this technology arouses interest in the most varied fields of science and

technology, such as those of pharmaceuticals, food processing, polymers, surfactants. SFE is a multidisciplinary technology, studied by chemists, engineers, and professionals of related areas [2]. Significant progress and achievements of SFE works have been reviewed in several recent articles [3,5].

Table 1 Summary with the advantages and disadvantages of greener extraction methods (Adapted from [3]).

Methods	Advantages	Disadvantages
Microwave-assisted extraction (used with traditional methods)	rapid extraction; small amount of solvent; relatively low additional costs; large number of compounds extracted;	use of high temperature; limited amount of sample; non-selective;
Pressurized fluid extraction (PFE)	small amount of solvent; improved extraction yield; rapid extraction; automated process;	use of high pressure; use of high temperature;
Supercritical fluid extraction (SFE)	rapid extraction; no solvent residue; preserves thermally labile compounds; tunable solvent (SCF) density; selective extraction; inexpensive to operate/run;	use of high pressure; high setup cost; technical knowledge of SCF properties required (e.g., phase behaviour);
Ultrasound-assisted extraction (used with traditional methods)	rapid extraction; small amount of solvent; relatively low additional cost;	non-selective;

1.2 Supercritical extraction

1.2.1 Properties of supercritical fluids

A supercritical fluid is defined as a substance above its critical temperature and pressure. This occurrence was first reported by Baron Cagniard de la Tour in 1822. He noticed that the limits between liquid and gas vanished once the temperature of certain materials was raised inside a closed flask [4]. Only in the late 70s, in Germany, did SFE start being utilized on an industrial scale, with the removal of caffeine from coffee [4]. Nowadays, industries worldwide adopt this sustainable process that does not require the use of organic solvents, or uses only small amounts of (usually) an alcohol as co-solvent. The extraction of compounds from natural sources is the most widely studied application of supercritical fluids (SCFs), with many scientific papers published [6].

Each substance has its own critical point. At this point, both liquid and gas densities become identical [7]. Supercritical fluids have gas-like diffusivity and viscosity, which facilitate permeation of solid matrices. They also exhibit liquid-like densities, which ensures solvation ability. Close to the critical point, small changes in pressure or temperature lead to significant changes in the density of a SCF, which allows for fractionation [3,8]. The ability to dissolve certain compounds preferentially from mixtures makes SCFs selective [1].

Figure 1 shows a typical pressure-temperature diagram highlighting the supercritical region. As seen in **Table 2**, it is noticeable that by increasing the pressure to four times that of the critical pressure (p_c) of an SFE, the density of the fluid can almost double, while it still maintains the diffusivity and viscosity typical of a gas.

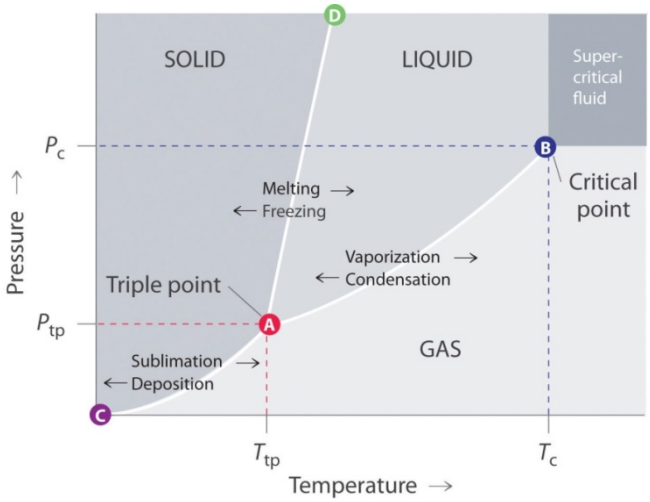


Figure 1 Typical schematic pressure–temperature phase diagram of a single component showing the SCF region [83].

Table 2 The relative properties of liquid, gas and SCF phases (Adapted from [3]).

	Physical Property		
Phase	**Density x 10^2 ($kg.m^{-3}$)**	**Diffusivity x 10^{-3} ($cm^2.s^{-1}$)**	**Viscosity x 10^{-4} ($kg.m.s^{-1}$)**
Liquid	6-16	<0.005	2-30
SCF: p_c,T_c	2-5	0.7	0.1-0.3
SCF: 4p_c,T_c	4-9	0.2	0-3-0.9
Gas	0.006-0.02	0.1-0.4	0.1-0.3

Like all processes, SFE has some drawbacks. The main limitation of SFE is the high cost of the initial investment on equipment and the energy needed to achieve the operation conditions [4], [7,9]. However, the amount of energy can be reduced by adding a co-solvent and the cost of the equipment is profitable in the long-term [7]. In fact, some studies have shown the economic viability of the SCF extraction of medicinal and aromatic compounds [9]. SCF is a very good choice when the product has a high value and when the SFE process yields a product with superior properties relative to a conventional process.

1.2.2 Types of supercritical fluids

Several substances have been studied with the purpose of evaluating their utility as a SCF in the extraction of natural produts. However, the majority of them was not adopted as a solvent of choice, due to toxicity, reactivity, the possibility of an explosion, damage to the environment, high cost or extreme supercritical conditions [9]. **Table 3** lists physical properties for various supercritical fluids [3].

In order to choose a supercritical fluid as a solvent, several aspects must be taken into consideration, including the properties of the fluid as a solvent but also economical and safety aspects [7], such as:

- Commercial availability;
- Need for co-solvents;
- Diffusivity;
- Ease of purification;
- Solvation ability;
- Heat and mass transfer parameters;
- Price (< 0.15 €/ Kg ideally);
- Toxicity;
- Environmental impact;
- Flammability;
- Proximity of p_c, T_c, to ambient conditions;
- Density close to p_C;
- Solubility of the solute in the SCF;
- Viscosity;

Taking these aspects into account, the compounds most commonly used as SCFs are carbon dioxide, water, acetone, alcohols such as ethanol and methanol, alkanes such as ethane, or unsaturated hydrocarbons such as ethylene [7].

Table 3 Physical properties of various supercritical fluids (Adapted from [4]).

SCF	Molecular Weight ($g \cdot mol^{-1}$)	T_c (°C)	p_c (bar)	Density at critical point ($kg \cdot m^{-3}$)	Notes
Air	n/a	−140.6	37.7	319.9	Green technology fluids and relatively high critical point densities;
Ammonia (NH_3)	17.03	132.2	113.3	225	
Nitrogen (N_2)	28.01	−147	34	313.3	
Water (H_2O)	18.02	373.9	220.6	322	
Carbon dioxide (CO_2)	44.01	30.9	73.7	467.6	Green technology and high critical point densities;
Xenon (Xe)	131.3	16.6	58.4	1110	Green technology and high critical point density but very expensive;
Chlorotrifluoromethane ($CClF_3$)	104.5	28.8	38.8	582.9	High critical point densities but environmentally hazardous;
Dichlorofluoromethane ($CHCl_2F$)	102.9	178.3	51.8	526.1	
Octafluoropropane (C_3F_8)	188	71.9	26.8	629	
Acetone (C_3H_6O)	58.08	235.1	46.4	278	Lower critical point densities and environmentally hazardous;
Benzene (C_6H_6)	78.11	289	49	30.9	
Dimethyl Ether ($(CH_3)_2O$	46.1	127.1	53.4	277	
Ethane (C_2H_6)	30.07	32.2	48.7	206.2	
Ethanol (C_2H_5OH)	46.07	240.9	60.6	276	
Ethylene (C_2H_4)	28.05	9.2	50.4	214.2	
Methane (CH_4)	16.04	−82.6	45.9	162.7	
Methanol (CH_3OH)	32.04	239.4	81	275.5	
n-Propane (C_3H_8)	44.1	96.7	42.5	220.5	
Propylene (C_3H_6)	42.08	91.9	45.5	230.1	

1.2.3 Supercritical CO_2 ($scCO_2$)

Supercritical carbon dioxide is the most popular supercritical fluid ($scCO_2$). It is the most commonly used SCF solvent for several reasons, namely its low critical temperature (T_c = 31.1 °C) and pressure (p_c = 73.8 bar), which allows to easily reach the supercritical fluid domain. This means it can be used for extraction of thermolabile substances without degradation [1,6,10] (**Figure 2**).

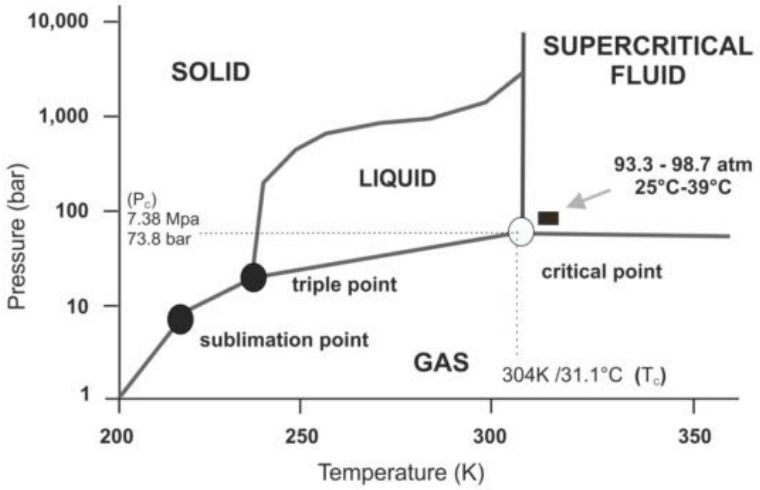


Figure 2 Schematic pressure–temperature phase diagram of CO_2 [8].

Also because the carbon atom of CO_2 is at its maximum oxidation number when combined with oxygen, CO_2 is inert towards further oxidation (i.e., non-flammable) [8,11]. Furthermore, being a highly chemically stable substance, there is no risk of secondary reactions, such as oxidation, reduction and chemical degradation [4]. In addition, it is safe, readily available, non toxic (it is allowed in the food industry), and easy to recycle. This lowers the cost of operation, and protects the environment. By being a gas at ambient conditions, CO_2 can be easily removed from the extracts, leaving no residues, which makes it the solvent of choice for the production of high added value products [4,6,7,9–12].

By being an apolar substance, CO_2 has a polarity similar to that of pentane and hexane, which are used mostly in traditional extraction processes [4,9]. Therefore, small non-polar or low polarity organic compounds, such as monoterpenes and sesquiterpenes, dissolve well in $scCO_2$. Slightly bigger and polar compounds, such as phenolic acids and flavonoids, can usually be dissolved (**Figure 3**). However, bigger and more polar compounds, such as sugars, polysaccharides, proteins, and tannins, are hardly soluble in pure supercritical CO_2 [9]. Typically, to overcome this problem, a co-solvent is added, selected in order to provide increased molecular interactions between CO_2 and the compounds of interest (Table 4) [3,9].

The most widely used modifiers are methanol and ethanol, which undergo dipole–dipole interactions and hydrogen-bonding with polar functional groups [13].

$ScCO_2$ extraction has replaced traditional industrial extraction techniques with selective separation of bioactive or thermally sensitive components from natural matrices for various practical purposes. More specifically, the increased use of plant extracts by the food, cosmetic, and pharmaceutical industries has made the extraction of essential oils using $scCO_2$ an attractive technology compared to conventional processes, such as organic solvent extraction and steam distillation, with respect to the product quality. CO_2 is generally recognized as safe (GRAS) by the FDA ("Food and Drug Administration") and the EFSA ("European Food Safety Authority") [2,13]. It also attracted a lot of attention in research and technology due to its "green" properties [9]. Consequently, the extraction of target compounds from a broad number of materials by $scCO_2$ has been extensively studied, as reflected by several thousands of published scientific papers.

Table 4 Co-solvents used in supercritical fluid extraction (SFE) of antioxidant compounds (Adapted from [78]).

Cosolvent	Class of Compounds	Number of times the yield increases compared to the yield with pure CO_2
Ethanol	Polyphenols	14.0
Ethanol	Flavonoids	2.2
Ethanol	Phenolic Acids	2.4
Ethyl Acetate	Phenolic Acids	3.8
Methanol	Phenols	4.0
Vegetable oils	Carotenoids	3.0 - 3.7
Water	Phenols	25.0

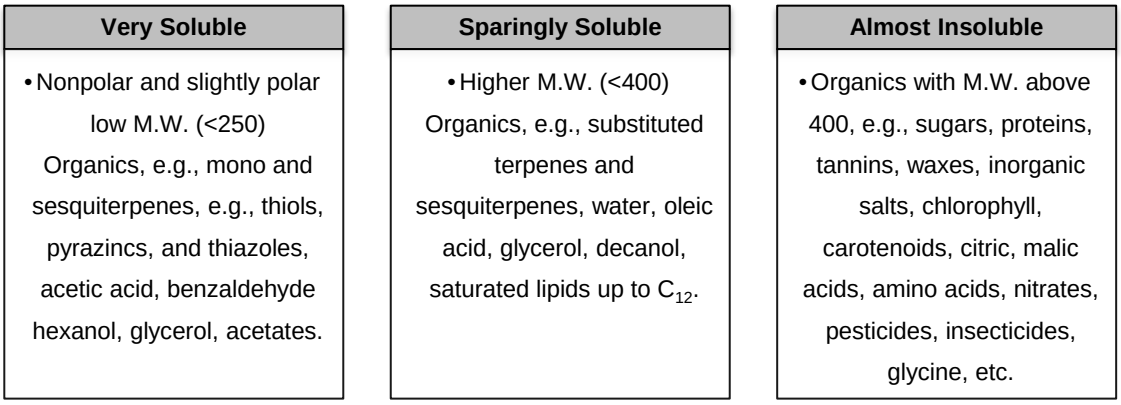


Figure 3 Solubility of botanical ingredients in $scCO_2$ (Adapted from [14]).

1.2.4 Parameters influencing supercritical extraction

In view of engineering processes and applications, there are two major factors affecting the development of the $scCO_2$ extraction technologies: the rate of mass transfer of the solute out of the material matrix, and the solubility of the extracted solute in the SCF [5]. The crucial parameters controlling the SCF extraction process are pressure and temperature, and how changes in these two paramaters affect SCF properties [3]. Nevertheless, additional parameters such as modifier content, solvent flow rate and residence time become a very important aid in applying this technology to chemical processes [5,11].

Some variables that should also be taken into consideration are the pre-treatment of solid matrices, such as drying, milling and grinding, particle size, particle size distribution, particle geometry and real density of solid particles. Other variables are the physical characteristics of the matrix extraction bed, including bed porosity, bed height, bed diameter, and initial content of target species in the solid matrix [5]. Theoretical models help to better understand and optimize the SFE [5,12,15].

1.2.5 Applications

The use of SFE has expanded in the natural products field in the last 20 years [3]. As mentioned earlier, SFE can be used in large-scale applications in the food, chemical processing, pharmaceutical, and cosmetic industries [4,16]. SFE has been used to obtain a large range of extracts from oils (edible/nutraceutical from plants and animals), oleoresins (for high quality condiments) and groups of bioactive compounds (alkaloids, terpenes, and phenolic), as well as single compounds (α-humulene, lycopene and α-tocopherol) [3,4,12].

Again, $scCO_2$ has been a solvent of choice, either pure or with polar modifiers for the extraction of moderately polar to polar natural products [16]. The extraction of essential oils from aromatic plants has been exploited by the pharmaceutical industry [4,6]. For example, the Roche company uses $scCO_2$ for the production of pharmaceutical products and intermediates [7]. In the food industry, $scCO_2$ has been used in the rectification and deodorization of edible oils and the extraction and fractionation of seed oils and fatty acids. It is also used in the extraction of lipids from cyanobacteria, of lycopene from tomatoes, of carrot carotenes, of bitter principles from hops, of essential and pungent principles from spices, of natural food colorants. Aditionally $scCO_2$ has been used in the decaffeination of coffee beans, the extraction of pure lecithin from crude lecithin, and separation of cholesterol from egg yolk, animal fats and meats [4,6,16], the extraction of natural plant insecticides [4]. Other applications in the chemical processing industries include the use of SFE for the separation of aromatics from petroleum products, the purification of polymers, the extraction of liquid hydrocarbons from coal and the removal of nicotine from tobacco for the production of light cigarettes [4].

1.3 Essentials Oils

According to the ancient Egyptian hieroglyphics and Chinese manuscripts, priests and doctors have been using essential oils for thousands of years to treat illnesses. Therefore they are known by man as the oldest medicines and cosmetics [4]. Essential oils are also known as volatile oils, because they are extracts of volatile fractions of plant origin [9,17]. The volatile denomination is justified by the fact that they evaporate rapidly when exposed to air at room temperature [4]. Essential oils are found in the form of small droplets between the cells of various parts of plants, namely in the aerial parts (leaves and thin branches), bark, trunks, roots, fruits, flowers, seeds and resins [4,17]. Although they are only a small fraction of the total amount of a plant, the compounds present in the essential oils are responsible for the characteristic aroma of each plant material [9]. Most often, essential oils are oily-looking fluids. However, some are solid at room temperature, such as camphor.

An essential oil can have more than one hundred components [4]. Compounds of essential oils can be divided into terpene (monoterpenes, sesquiterpenes) and non-terpene oils (phenylpropane derivatives) [4,18]. The first extraction processes used to obtain essential oils were the digestion of flowers and condiments in oils and fats, also called enfleurage. However, it became necessary to develop other processes to obtain essential oils at an industrial level. These methods, already mentioned, include SFE [4,17]. The major components of essential oils, the terpenes, are easily dissolved in dense CO_2 [15]. Essential oils are products of high added value, but some components of these complex mixtures have low thermal stability [4]. Generally, SFE is used as an alternative to processes using high temperatures or liquid solvents [19].

The use of essential oils occurs primarily in the pharmaceutical and food industries, but also in the cosmetics, perfumery, hygiene and cleaning industry. In aromatherapy, oils are ingested both pure and diluted in alcohol, and even in medical preparations. Externally, they can be used in frictions, massages and inhalations [4].

1.4 Carqueja

1.4.1 Botanic characterization

Carqueja is a common name given to the species *Pterospartum tridentatum (L.) Willk* (=*Chamaespartium tridentatum, Genista tridentata*). It is a small shrub belonging to the Leguminosae (=Fabaceae) family and Papilionoideae (=Faboideae) subfamily [20]. It belongs to the Fabales order, of the Rosidae sub-class and the Magnoliopsida class. We can find three subspecies of *P. tridentatum: Cantabricum*, *Tridentatum* and *Lasianthum* (**Figure 4**) [21].

In the literature, the herb has different names, such as Carquejila, Carqueijeira, flower-of-carqueija, carqueixa, carquesa and carquesia. These variations of the name derived from errors, divergent phonetics and linguistic pronunciation [22].

Distinctive species sometimes are designated by the same common name. Such is the case of Carqueja that corresponds in mainland Portugal to *Pterospartum tridentatum*. In Madeira Island, the same name refers to a different species of the same family, namely *Gorse* (*Ulex europaeus*). The Carqueja, in Brazil, is a totally different species - *Baccharis trimera* from the Asteraceae family [23].

P. *tridentatum* is a shrub that grows spontaneously up to 100 cm, prostrate or erect. Its branches are 2-14 mm wide, with 5-6 flattened and wavy backs, two of which resemble opposite wings. The leaves are more or less developed (unifoliate); the stipules are shaped like triangular teeth, sharp and sometimes curved. The limb is triangular, straight or curved, similar to and closely attached to the stipules, resulting in a trident-shaped leaf (**Figure 6**) [20,24]. The inflorescences are formed by 3 to 10 yellow flowers with a typical odor, traditionally harvested during Springtime, from April, in low-lying areas, and from May to July, in the lands at higher altitude (**Figure 4** , **Figure 5**) [25].

Figure 4 Carqueja flowers [84].

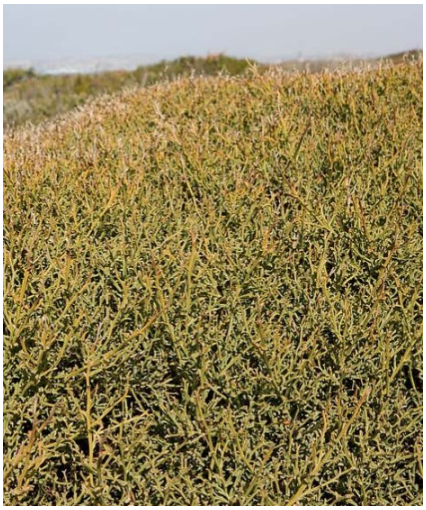
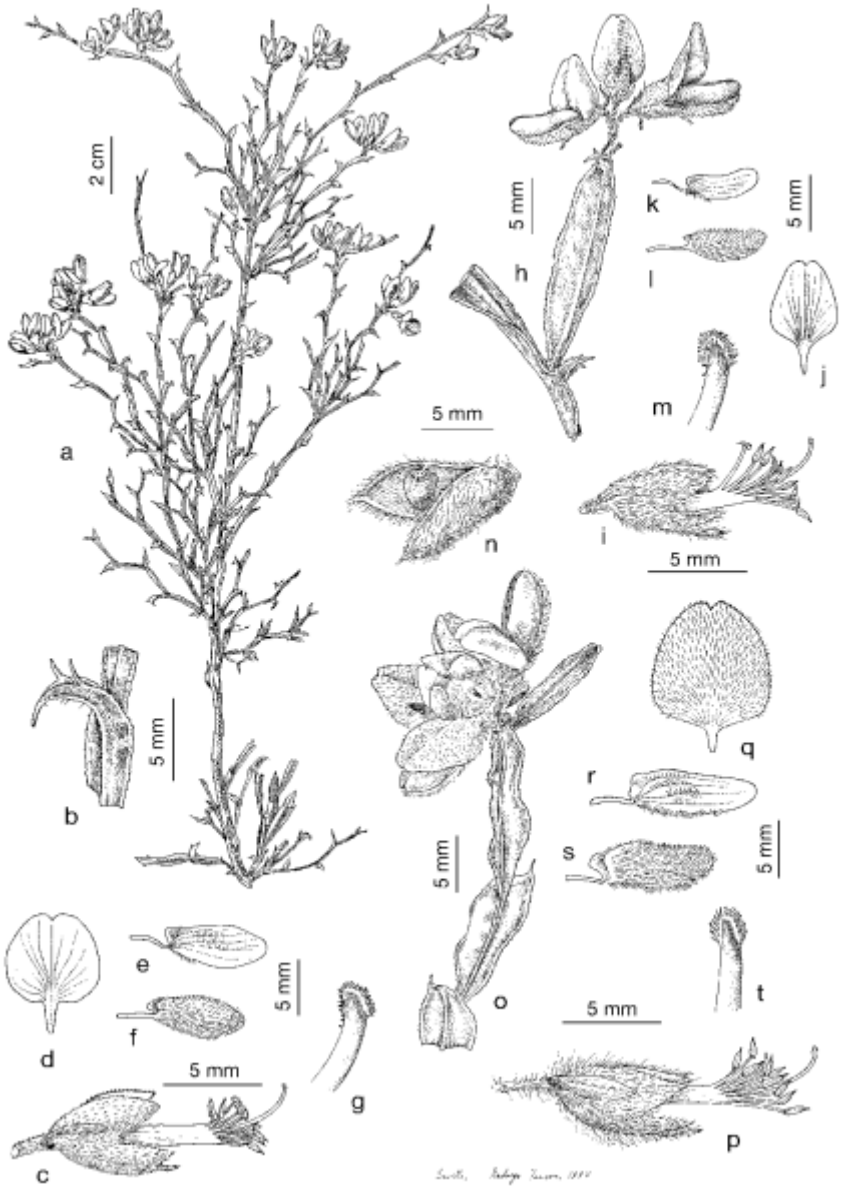


Figure 5 Carqueja out of the flowering season [84].



A – Habit
B – Knot with leaf
C – Flower in the anthesis, without corolla
D – Standard
E – Wing
F – Keel
G – Stigma
H – Detail of a floriferous branch
I – Flower in the anthesis, without corolla
J – Standard
K – Wing
L – Keel
M – Stigma
N – Fruit with seed
O – Detail of a floriferous branch
P – Flower in the anthesis, without corolla
Q – Standard
R – Wing
S – Keel
T – Stigma

Figure 6 *Pterospartum tridentatum* subsp. *tridentatum*, Torres Vedras, Estremadura (SEV 3577): a) b) c) d) e) f) g); P. *tridentatum* subsp. cantabricum, h-m) Foncebadón, León (SEV 73168);
n) Serra do Gerês, Minho (SEV 133523): h) i) j) k) l) m) n); P. *tridentatum* subsp. lasianthum, Los Barrios, Cádiz (SEV 140825): o) p) q) r) s) t); (Adapted from [24]).

1.4.2 Geographic distribution

Carqueja is an autochthonous plant of the Northwest part of the Iberian Peninsula and North of Africa (Morocco) [26]. *P. tridentatum* is an endemic species, common in the mountains of the north of Portugal and Spain. It can usually be found in the understory of *Arbutus unedo*, *Pinus pinaster,* and *Eucalyptus* forests but it can also be found in abandoned lands with acidic soils, brushwoods and thickets [26,27].

1.4.3 Phytochemical compounds

The first chemical composition report of the P. *tridentatum* species appeared in 2004. The most traditional extraction process was used: water extraction from the dried flowers of P. *tridentatum*. Vitor *et al.* referred that the main constituents were three derivatives of genistein (4',5,7-trihydroxy- isoflavone), namely the isoflavones sissotrin, genistin and prunetin. In addition, it was also identified one new isoflavone, the 5,5'-dihydroxy-3'- methoxy-isoflavone-7-O-β-glucoside, and the flavonol glycoside isoquercitrin [28]. However, in recent studies, it has been possible to reveal the presence of other phenolic compounds, as well as essential oils and alkaloids in this plant. Phenolic compounds are the dominant chemical compound group [29]. **Table 5** shows the attempts made for the identification of compounds present in P. *tridentatum*, found in the literature.

Table 5 Identification of compounds, depending on the type of extraction, of P. *tridentatum*.

		Phytochemicals	Part of plant	Type of extracion	REF
Flavonoids	Chalcone	Phloretin	Flowers	Ethanolic and Aqueous	[30]
	Flavanone	Taxifolin-6-C-glucoside	Flowers and S&L	Aqueous (Soxhlet)	[31]
	Flavone	Apigenin, Biochanin A-glucoside, Isorhamnetin-O-hexoside, Luteolin-O-(O-acetyl)-glucuronide and Luteolin-O-glucuronide	Flowers	Methanolic and Water	[26,32]
	Flavonol	Dihydroquercetin 6-C-hexoside, Myricetin, Myricetin-6-C-glucoside, Pentahydroxy-flavonol-di-Oglucoside, Quercetin 3-O-galactoside, Quercetin deoxyhexosyl-hexoside, Quercetin O-hexoside, Quercetin-3-O-glucoside (isoquercitrin), Quercetin-3-O-rutinoside (rutin) and Taxifolin	Flowers and S&L	Infusion, Methanolic, Ethanolic and Aqueous (Soxhlet)	[28,30–36]
	Isoflavone	4'-O- Methylgenistein (biochanin A), 5,5'-Dihydroxy-3'-methoxyisoflavone-7-O-β-glucoside, 7-O-Methylgenistein (prunetin), 7-O-methylorobol, Biochanin A 7-O-glucoside (sissotrin), Biochanin A O-acetylhexoside-O-hexoside, Biochanin A O-hexoside-O-hexoside, Genistein, Genistein 7-O-glucoside (genistin), Genistein-8-C-glucoside Biochanin A O-hexoside, Methylbiochanin A/methylprunetin and Ohexoside	Flowers and S&L	Infusion, Methanolic and Aqueous (Soxhlet)	[28,31–36]
Hydroxy benzoic acid		Ellagic, Syringic and Vannilie acid	Flowers and S&L	Methanolic	[37]
Hydroxy cinnamic acid		Caffeic, Chlorogenic, Ferulic, Malic, p-Coumaric and Rosmarinic acid	Flowers and S&L	Infusion and Methanolic	[26,32,34,37]
Tocopherols		α- (vitamin E), γ- and δ-Tocopherol	Flowers	Infusion and Methanolic	[34]
Sugars		Arabinose, Fructose, Fucose, Galactose, Galacturonic Acid, Glucose, Mannose, Rhamnose, Sucrose and Xylose	Flowers	Infusion, Methanolic, Ethanolic and Aqueous (Soxhlet)	[34,30]
Organic acids		Citric, Oxalic, Quininic and Shikimic acid	Flowers and S&L	Infusion, Methanolic and Aqueous (Soxhlet)	[34,31]

A study on the essential oil from the aerial parts of P*. tridentatum* (L.) Willk. collected during the flowering phase showed cis-theaspirane (13%), trans-theas-pirane (12%) and octen-3-ol (11%) to be the main components [38]. **Table 6** shows the composition of the essential oils isolated in extracts obtained by distillation-extraction using *n*-pentane as solvent, from the various parts of Carqueja. These were collected during the flowering phase, in different years (2002 to 2004) and in different places (Ribatejo and Beira Alta).The chemical variability present in the essential oil of *Pterospartum tridentatum* (L.) Willk. allowed the oil to be considered as an alternative to chemical preservatives [31].

Table 6 Composition of the essential oils isolated by distillation-extraction from various parts of *Pterospartum tridentatum* (Adapted from [38]).

Components	Flowers	S&L	Aerial parts
trans-2-Hexenal	x	x	x
cis-3-Hexen-1-ol and cis-2-Hexen-1-ol	x	x	x
n-Hexanol and *n*-Heptanal	x	x	x
n-Nonane	-	x	x
Benzaldehyde	x	x	x
α-Pinene	x	x	x
n-Heptanol	x	x	x
1-Octen-3-ol	x	x	x
2-Pentyl furan	x	x	x
3-Octanol	x	x	x
Benzyl alcohol	-	x	x
Benzene acetaldehyde	x	x	x
1,8-Cineole	x	x	-
Limonene	x	x	x
Acetophenone	x	x	-
n-Octanol	x	x	-
Heptanoic acid	x	x	x
Phenyl ethyl alcohol	x	x	x
n-Nonanal	x	x	x
Linalool	x	x	x
cis-Rose oxide	x	x	-
n-Undecane	-	-	x
trans-Rose oxide	-	x	x
trans-Pinocarveol	-	-	x
2- trans,6 cis-Nonadienal	x	-	-
2- trans-Nonen-1-al	x	x	-
Pentyl benzene	x	x	-
Octanoic acid	x	x	-
α-Terpineol	-	-	x
Safranal	x	x	-
n-Decanal	x	-	-
Geraniol	x	x	x
n-Decanol	x	x	x
Perilla alcohol	-	x	x
Nonanoic acid	x	x	-
cis-Theaspirane	x	x	x
2 trans,4 trans-Decadienal	x	x	x
trans-Theaspirane	x	x	x
Eugenol	x	x	x
α-Copaene	-	-	x
β-Bourbonene	-	-	x
Longifolene	-	-	x
β-Caryophyllene	x	-	x
Geranyl acetone	x	-	x
Germacrene-D	x	-	x
ϒ- and δ-Cadinene	x	-	x
Dodecanoic acid	x	x	x
β-aryophyllene oxide	-	-	x
n-Tetradecanal and *n*-Pentadecanal	-	-	x

1.4.4 Properties and applications

P. *tridentatum* is one of the most widely used medicinal and aromatic species in Portugal since ancient times [39]. Thus it is also one of the most cited Portuguese plants in ethnobotanical studies referring to the Portuguese flora, not only by abundance but also by the numerous uses [40]. In traditional medicine, this species has a wide application in the human body in the form of infusion, poultice and syrup [30]. It is known for having digestive, diuretic, purgative, diaphoretic, hypotensive, hypoglycemic, analgesic, antibiotic, anti-inflammatory, antioxidant, antispasmodic, antiseptic, healing and soothing properties. The literature refers countless purposes of treatments and health benefits [20,22,27,28,33–35,32,31,30,38,40–43]. One study of 364 plants reported 52 different uses of Carqueja, which supplanted all other plants in the number of applications [22].

In particular, the infusion of the dry flowering of P. *tridentatum* is used for febrile, rheumatic, gastric, intestinal, hepatic, biliary, urinary and respiratory tract conditions. It is recommended as well against asthenia, for heart conditions, headaches and migraines [22,30,43]. This infusion is taken for the control of uric acid in gout, and for diabetes. Carqueja flowers are one of the ingredients of several herbal blends sold in Portugal for the treatment of diabetes. Since Carqueja is rich in phenolic compounds and the increase in free radicals appears to be responsible for some of the complications of diabetes, especialy those affecting vascular tissue, treatment with antioxidants is one of the recommended treatments [41]. It is used to lower cholesterol by its hypoglycemic and antihypercholesterolemia characteristics. It is an excellent emollient, a sedative, but was also used externally for wound healing and for skin inflammations and infections [22,38,40,43]. Some authors have shown that P. *tridentatum* has important phenolics often associated with anti-inflammatory, antioxidant, and antimicrobial propertie [32]. The infusion of P. *tridentatum* had a very high content of phenols and flavonoids and stood out in most antioxidant activity trials. Cytotoxicity studies have been carried out on this species involving reduction capacity or scavenging capacity of reactive oxygen species (ROS), suggesting the involvement of these compounds in this type of antioxidant activity. ROS appear to be implicated in several degenerative diseases as well as in the aging process [41]. *P.tridentatum* presents similar antifungal effects, being effective against *Candida* strains belonging to *C. albicans, C. Glabrata* and *C. parapsilosis* species [44]. The anti-inflammatory activity of methanolic extracts of P. *tridentatum* was determined, and it was concluded that the extracts of stems, leaves and flowers have the capacity to decrease the initial speed of the enzymatic activity of the enzyme 5-LOX [31]. Isoquercetin, extracted from Carqueja, protected the cells of the veins of the human umbilical cord when damaged with induced H_2O_2, revealing antioxidant protection [30].

Although the amin uses of Carqueja fall in the medicinal category, this plant is also widely used for culinary and spice purposes. Leaves and stems are usually used in cooking, to enhance the flavor of rice roast meat or hunting animals [26,40]. Addicionally, the leaves are used in fresh

salads as condiment and the stems are used to prepare dishes like "arroz malandro" or "malandrinho" [22,45]. Carqueja is also used to make traditional dishes that are part of the menu of many local restaurants, such as “arroz de Carqueja” [31]. Additionally, dried stems are used as firewood in traditional ovens because they are highly combustible, giving an enjoyable aroma to bread, and their combustion releases a pleasant odor, used to aromatize the house [26].

Carqueja can also be used for bioethanol production for being an economical and readily available substrate. The woody biomass is mainly composed of cellulose, hemicellulose and lignin. The application of these residues to bioprocesses is favorable due to the fact that they are collected in the cleaning of the forests for fire prevention [22]. It has also been reported the use of Carqueja as pasture for goats and sheep. All the aerial part is used to make the cattle beds in the pens. The decomposition of the plant material with excreta forms the manure used as fertilizer in vegetable gardens, and in cereal fields. The flowers are used in hives to attract bees, and later, with the smoke from the burning, the hives are fumigated to remove the honey [22]. The study of “Ethnobotany of Northeast Portuguese: knowledge, plants and uses” refers to the use of Carqueja in the village of Montesinho Natural Park as fuel, medicine, spice and animal feed [31]. It has been concluded that the flowers are innocuous and can be used in cooking or in traditional medicine for the treatment of diseases associated with oxidative stress, at least for short-term therapies [31].

Carqueja is an underexploited natural source of compounds with biological activity, which should be fully characterized aiming to its valorization [46]. Its properties are so many that the tea of the flowers of P. *tridentatum* is commonly used in Portugal as a panacea, being considered as a potential cure for all illnesses of the body [26].

1.5 Waltheria Indica

1.5.1 Botanic characterization

Waltheria indica L. or *Waltheria americana L.* is the scientific name for a shrub belonging to the Malvaceae family and Sterculiaceae subfamily [47], also known as a sleepy morning, velvetleaf, marsh-mallow, monkey bush, boater bush, leather coat, buff coat, and by many other names.

W. *indica* was reported in 1753 by Linnaeus [48]. Some authors considered W. *indica* as part of the Sterculiaceae family [49]. However recent studies in phylogeny have shown that the distinction of these families is unsustainable. For this reason, the Malvaceae family, according to the Angiosperm Phylogeny Group (APG II), includes the Sterculiaceae, Tiliaceae and Bombacaceae families, each being maintained at the subfamily level [50]. The *Waltheria L*. genus is represented worldwide by around 60 species [51].

W. *indica* is a short-lived shrub or subshrub, where the stem diameter can reach 2 cm and the height up to 2 m. [48].This plant develops a weak taproot, robust lateral roots and abundant fine roots. The roots are flexible and have a brown color. This shrub generally has a single, tough stem emerging from the ground, but frequently branches near the ground. W. *indica* usually has an upright and somewhat branchy form. However, in some environments, it may grow in a semi-prostrate habit (**Figure 8**). The budding leaves and stems are covered with a gray, velvety pubescence.

The alternate leaves are narrowly ovate or oblong with a rounded to a subcordate base, irregularly serrated edges and a normally rounded tip. The blades range from 2 to 12 cm long and 1 to 7 cm wide, and the petioles range from 0.5 to 3.3 cm long (**Figure 8**). Axillary inflorescences are usually dense glomerules that contain until 22 yellow to orange fragrant flowers (**Figure 7**) [52]. The seeds are very small, black and have an obovoid shape, in each 2 mm capsule [53]. Flowering and fruiting occur essentially in October [54].

Waltheria indica is recognized by the absence of glandular trichomes, both in the branches and in the leaves, and unlike the other species of the Waltheria genus, it has subserseal flowers. It was verified that this species presents great morphological variability, besides having homostylous flowers, which is a rare character found in the genus [54]. The plant blooms after 6 months of growth and its flowering (**Figure 7**) is more or less continuous until its death [48]. Death usually occurs during the dry season [53].

Figure 7 Waltheria *indica* flowers [85].

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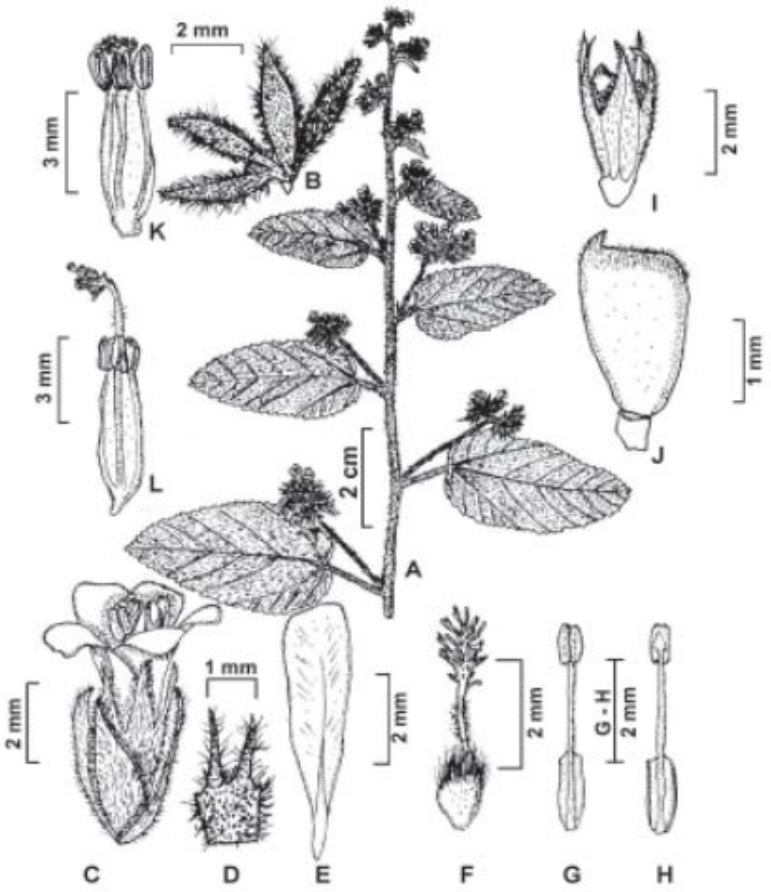


Figure 8 *Waltheria indica L*. A) Branch with flower and fruit. B) Bracts, front view. C) Flower brevistila. D) Detail of the chalice, abaxial face. E) Petal, adaxial face. F) Gineceo. G and H) Stamens, abaxial and adaxial. I) Chalice covering the fruit. J) Fruit. K) Detail mediastila flower. L) Longistila flower detail [51].

1.5.2 Geographic distribution

W. *indica* was apparently naturalized in Hawaii soon after the arrival of colonists. The species is native to the New World where it occurs from Florida and Texas to Brazil [49]. W. *indica* develops throughout the tropical and warmer subtropical zones [53]. This species is the only of its kind with pantropical distribution in that it is present in all the tropical regions of Africa, Asia and the Americas (**Figure 9**) [54]. In the American continent, it is distributed from the southern United States to the Central region of Argentina, being frequent in open areas of secondary vegetation, in plantations, roadside borders, rocky fields and coasts [52]. In Hawaii, W. *indica* is known as Uhaloa and it survives in a diverse range of soils with igneous, sedimentary and metamorphic rocks. It perseveres in drought, salt spray and slightly salty soil. It does not tolerate a shade canopy and is unable to live in dense grasslands [55].

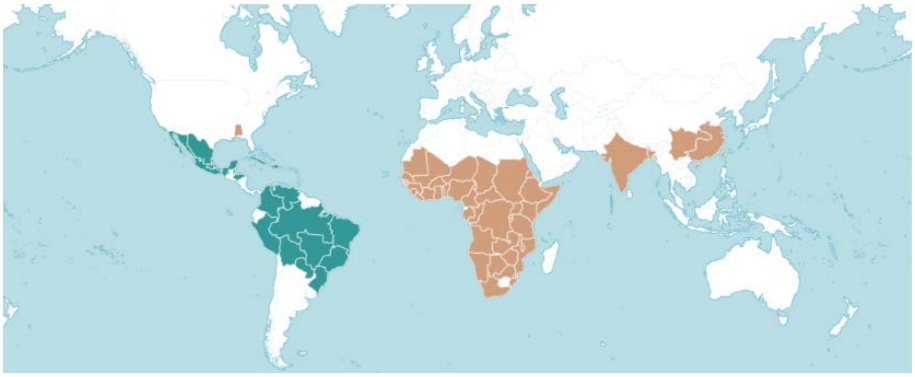


Figure 9 Global geographic distribution of the Waltheria *indica* L. species [86].

1.5.3 Phytochemical compounds

The phytochemical screening showed that the aqueous extract of stems with leaves of W. *indica* contained tannins, flavonoids, saponins, triterpenes, sterols and anthocyanosides [56]. However it is reported that W. *indica* also contains different chemical groups including alkaloids, cardiac glycosides, anthraquinones, carbohydrates and mucilages. The regularly occurring chemical constituents of W. *indica* belong to flavonoids [57]. **Table 7** shows the main compounds isolated from W. *indica* reported.

Table 7 Chemical groups, part of the plant studied and compounds isolated from *Waltheria indica* L. (Adapted from [57]).

		Phytochemicals	Part of plant	Type of extracion	REF
Phenolic compounds	Flavan-3-ols	(-)-Epicatechin	Whole plant	Ethanolic	[58]
	Flavone	5,2′,5′-trihydroxy-3,7,4′-trimethoxyflavone, 5,2′-dihydroxy-3,7,4′,5′-tetramethoxyflavone, Chrysosplenol E, Flindulatin, Hydroxy 3,4',7-trimethoxyflavone and Oxyanin A	Whole plant	Dichloromethane	[59,60]
	Flavonol	Gossypetin, Gossypetin-8-glucuronide, Herbacetin, Herbacetin-8-O-glucuronide, Kaempferol, Kaempferol-3-O-galactoside, Kaempferol-3-O-α-L-rhamnoside, Kaempferol-3-O-β-D glucopyranoside, Kaempferol-3-O-β-D-(6″-E-p-coumaryl)-glucopyranoside, Quercetin, Quercetin 3-O-glucopyranoside, Tiliroside and Vitexicarpin	Whole plant	Dichloromethane and Ethanolic	[58–60]
	Tannins	-	Whole plant	Aqueous, ethanolic, petroleum ether, ethyl acetate hexane and hydro alcoholic	[49,56,61–64]
Non-phenolic compounds	Alkaloids	(R)-Vanessine, 8-deoxoantidesmone, Adouetins: Y, Y', Z, X, Antidesmone, Waltherione: A, C, E, F, G, H, I, J, K, L, M (3-methoxy-2-methyl-5-(5-phenylpentyl)-5,6,7,8-tetrahydroquinolin-4(1H)-one), N (8-hydroxy-3-methoxy-2-methyl-5-octyl-5,6,7,8-tetrahydroquino-lin-4(1H)-one), O (2-(hydroxy-methyl)-3-methoxy-5-(5-phenylpentyl)-5,6,7,8-tetrahydroquinolin-4(1H)-one), P (3-methoxy-2-methyl-5-(5-phenylpentyl)-1,5,6,7-tetrahydroquinoline-4,8-dione) and Q ((5S,8R)- 3,8-dimethoxy-2-methyl-5-(5-phenylpentyl)-5,6,7,8-tetrahydro-quinolin-4(1H)-one)	Whole plant	Aqueous, ethanolic, petroleum ether, ethyl acetate, hexane and hydro alcoholic	[47,49,56,59–65]

	Anthraquinones	-	Leaves, stem and roots	Ethanolic	[47,49,64]
	Carbohydrates	-	Leaves, stem and roots	Aqueous, ethanolic and hexane	[47,56,62,64]
	Cardiac glycosides	-	Whole plant	Aqueous, ethanolic, petroleum ether, ethyl acetate and hexane	[47,49,61,62,64]
	Saponins	Tri terpinoildal saponins	Leaves, stem and roots	Aqueous, ethanolic, hexane and Hydro alcoholic	[47,49,56,62–64]
	Sterols	-	Whole plant	Aqueous, ethanolic, ethyl acetate and hexane	[47,56,61,62,64]
	Terpenes	Triterpenes, 3β- Acetoxy-27-cis-caffeoyloxyolean-12-en-28-oic acid methyl ester, 3β- Acetoxy-27-trans-caffeoyloxyolean-12-en-28-oic acid methyl ester and Betulinic acid	Whole plant	Dichloromethane, ethanolic, ethyl acetate and petroleum ether	[47,59,61,62,64,65]
New natural compounds		Methyl (2R,3R)-3,4-dihydro-3,8-dihydroxy-2- methyl-(4-methylpent-3-en-1-yl)-2H-1-benzopyran-6-carboxylate, Methyl (R)-2,3-dihydro-7-hydroxy-2-[(S)-2-hydroxy-6-methylhept-5- en-2-yl]-2H-1-benzofuran-5-carboxylate, Methyl (R)-2,3- dihydro-7-hydroxy-2-[(2R,5S)-5-(2-hydroxypropan-2-yl)-2-methylte- trahydrofuran-2-yl]-2H-1-benzofuran-5-carboxylate, (2 S)-2-[(1S)- 1-(5,5-dimethyltetrahydrofuran-2-yl)-1-hydroxyethyl]-2,3-dihydro-2H-1-benzofuran-5-carboxylic acid and (2 S,4aR,10aS)-2,3,4,4a,10,10a-hexahydro-6-hydroxy-2-(2 hydroxypropan-2-yl)-4a-methyl-pyra-no[3,2 b][1]benzopyran-8-carboxylate	Aerial parts	Dichloromethane	[59]

1.5.4 Properties and applications

Waltheria indica L. has diverse uses, mainly in medicine. This plant is widely used as an antioxidant, traditionally to treat a variety of human infections and to treat various central nervous system-related conditions, such as pain, neuralgia, seizures and sleep problems [47], [66].

The plant has been utilized as a decoction or infusion where emollient, febrifugal, purgative, tonic, analgesic and astringent action is sought [49]. It is used in several ethnomedical treatments, mainly for pain and inflammation (gingivitis, neuralgia, rheumatism and sore throat). It is also used in treatment of dysentery, diarrhea, wounds, conjunctivitis, asthma, cough, anemia, malaria, syphilis, hemoptysis, cancers, gastric ulcer, infertility and erectile dysfunction [57].

Several uses of this plant have been described in inflammation-related diseases around the world, such as rheumatisms and cancer in Mexico and conjunctivitis in Madagascar [59]. In Hawaii, the whole W. *indica* is defined as an “aspirin-like” traditional medicine for inflammations, and the root bark is chewed to treat sore throats and leprosy in humans [59]. It is also used as a treatment for headaches, asthma and vertigo [66]. In Panama, the stems are used as a chew stick while its extracts are used as an eye bath and a remedy for hemoptysis, treatment of cough and a cure for female sterility [49]. A study in Panama showed that W. *indica* possesses HIV protease inhibitory activity, an essential enzyme during the replicative cycle. Aqueous extracts from the branches showed the ability to inhibit their enzymatic capacity by 50% [48]. Leaves of W. *indica* are also consumed in Brazil as a tea against inflammation such as gingivitis [59].

W. *indica* is commonly used in Burkina Faso to treat malaria [65]. A study indicated that methanol extractions of W. *indica* are a good resource for antimalarial drugs [48]. In Niger/Nigeria, traditional healers give the whole plant to cattle as a tonic, suggesting a possible activity against Nagana [65]. It is used by the Hausas for the treatment of skin diseases, impotence, and infertility, as an aphrodisiac and as children’s medicine at birth and during teething. The aqueous extract of the root, in the Fulani community, is used for mitigating pains and aches during the “Sharo” festival. Among the Yorubas, the aqueous extract of the root and stem is used for treating syphilis, internal hemorrhage and as a restorative during harvesting activities [67]. In Togolese traditional medicine, it is used to treat diabetes mellitus [63], probably due to the presence of mineral elements in the W. *indica*’s root, such as Zn, Cu and Mn [64]. The root is also used in dysentery, diarrhea with ‘‘moco’’ (white mucus) [68]. In Tanzania, a report on extracts from several plants used for the treatment of infectious diseases against bacteria, viruses and fungi were evaluated. The results showed that the extractions with *n*-hexane from W. *indica* shoots have antibacterial activity. It has been recognized that W. *indica* can be useful for the treatment of diarrhea with blood, syphilis and seizures [48].

It is known from International patent application WO 98/55087 (Laboratoires Sérobiologiques) that the natives of the tropical primeval forests of South America use a decoction of Waltheria *indica.* They use it for ritual washing because it has an antiseptic, wound healing, astringent and invigorating effect. It is proposed, in the same document, to use the extracts of this plant for topical treatment of the skin, for example as depigmenting agents. Additionally, the extracts W. *indica* are recommended as antiviral preparations in European patent application EP-A2 0 568 001 [69].

The wide use of W. *indica* in folk medicine has been corroborated by scientific pieces of evidence in recent time, namely regarding antioxidant effects, antibacterial effects, antifungal effects, antimalarial effects, anti-inflammatory effects, analgesic effects, anticonvulsants effects, antidiarrheal effects, anti-anemic effects, effect on central nervous system and trypan genocidal effects of W. *indica* [57,67]. A pharmacological study focusing on aqueous extracts from the root and stem of W. *indica* suggested its analgesic potential. Furthermore, the ethanol or aqueous extracts from the whole plant proved to have anticonvulsant and sedative properties, which can make them useful for the treatment of neurological diseases [66].

In spite of so many medicinal applications, caution should be applied when using W. *indica* leaves, for the excessive use of the aqueous extracts can be hepatotoxic, as some studies have concluded [70]. However, the toxicity of aqueous extracts of this plant was considered to be low, according to the scale of Hodge and Sterner (1943) and the WHO [56].

1.6 Antioxidant Activity and Phenolic Compounds

Several substances of plant origin are of interest to the scientific community because of the presence, in their composition, of compounds with antioxidant capacity [2]. The antioxidant species can be defined as “any substance that delays, prevents or removes oxidative damage to a target molecule”, that is, as any molecule that inhibits the oxidation of another molecule [71]. Antioxidants are compounds known to react with free radicals and/or reactive oxygen species, in order to inactivate them, preventing oxidative damage and, consequently, cell wear [2].

The antioxidants can be classified in two ways. One of them distinguishes three categories: the primary antioxidants, which are involved in the prevention of oxidant formation, secondary antioxidants, known to be scavengers of ROS, and tertiary antioxidants that repair the oxidized molecules through sources like dietary or consecutive antioxidants.The second way distinguishes enzymatic or non-enzymatic antioxidants [71], as shown in **Figure 10. Figure 11** focuses on the important family of flavonoids.

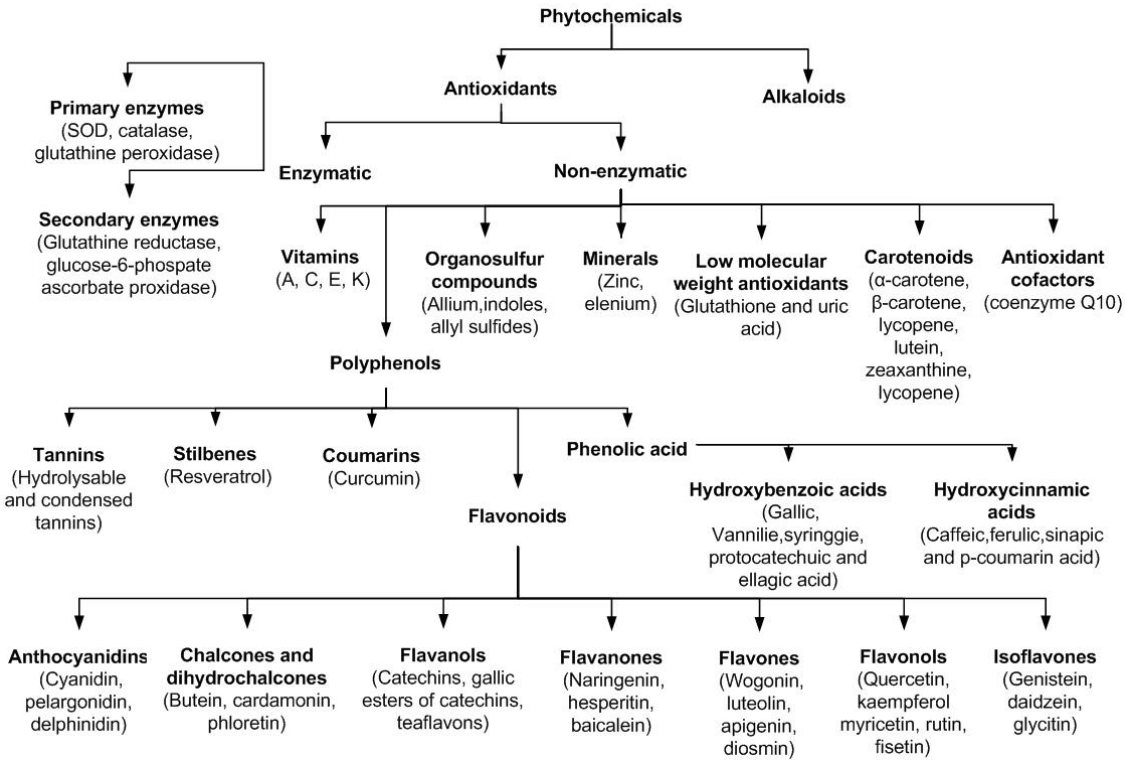


Figure 10 Phytochemicls schematic with major antioxidants and classification of phenolic compounds present in the plant system (Adapted from [87–90]).

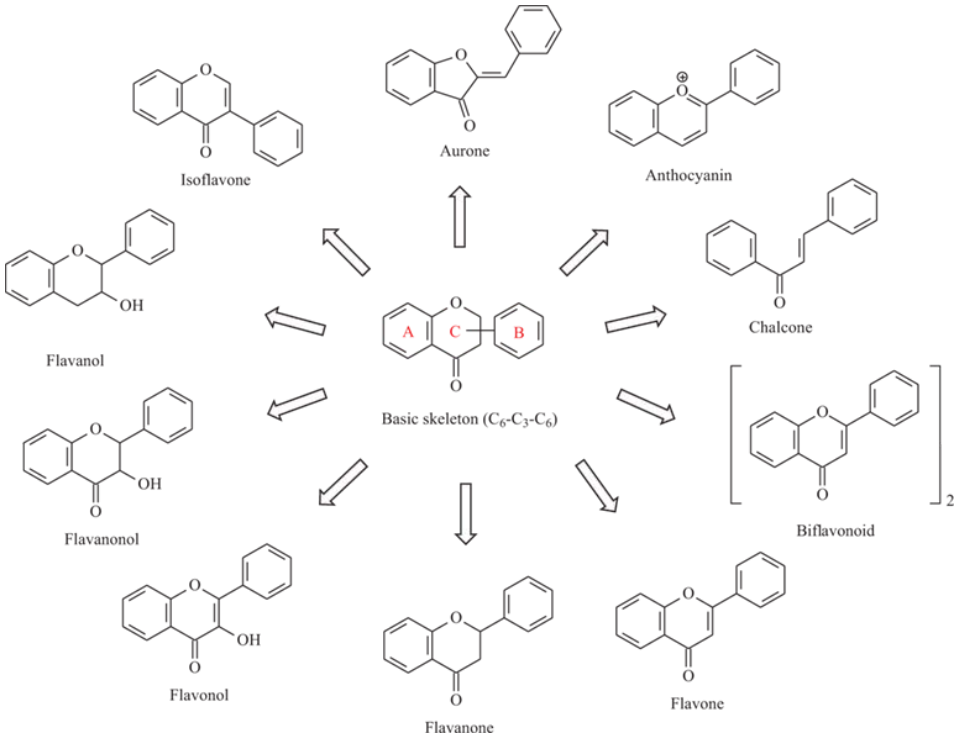


Figure 11 Basic skeleton structure of flavonoids (C_6-C_3-C_6) and all their classes [72]**.**

Flavonoids, one of the classes of polyphenolic compounds present in plants, have a very well studied mechanism of antioxidant action that consists in the capture of free radicals. This property depends on the chemical structure of the molecule in relation to the electron donating capacity and the stabilization of the radical formed by the relocation of the unpaired electron. This fact has been raising the interest of scientists and industries since they help to promote health care and protect against heart diseases and cancer [2]. It is reported in the literature positive findings for the interaction of antioxidants during cancer treatment [73]. The antioxidant activity of a compound is determined by factors such as reactivity with the electron donor, the potential to chelate transition metals, reactivity with other antioxidant compounds, and the fate of the antioxidant radical derivative. There are different methods of determining the antioxidant activity, some based on the capture of certain radicals, others based on the power of reduction of metals and the quantification of products f ormed during lipid peroxidation [13].

Several studies point to the imbalance between oxidants and antioxidants as the cause of many pathologies [2]. Many recent studies were made with the intent of exploring the effects of antioxidants in the prevention and treatment of various diseases, shown in **Table 8** [71]. Polyphenols generally named phenolic compounds are the group of secondary metabolites frequently found in the extracts of medicinal and aromatic plants and they are generally reported as having antioxidant and antimicrobial activity [32]. They have as a common feature at least one aromatic ring, in which at least one hydrogen is replaced by a free hydroxyl group or other derived function, such as ester. These compounds, in general, are very unstable molecules,

being readily oxidizable and susceptible to degradation. The main factors that can trigger their degradation are high temperatures, extreme pH values and the presence of light and oxygen [9]. Therefore an alternative to the traditional methods for their extraction is the SFE method, since it provides the extraction of relatively clean extracts, without the presence of sugars, chlorophylls, and pigments, at relatively low temperatures, which guarantees their stability [2,9].

Table 8 The efficiency of antioxidants in prevention and treatment of various diseases (Adapted from [71]).

Disease studied	**Antioxidant used**
Mortality: Primary/Secondary Prevention	Beta-carotene, vitamin A, vitamin C, vitamin E, and selenium
Fatty liver disease	Vitamin A, carotenoids, vitamin C, vitamin E and selenium
Amyotrophic Lateral Sclerosis (SLA)	Vitamin E 500 mg twice daily
Multiple Sclerosis	Omega-6 fatty acids (11-23 g/day linoleic acid)
Alcoholic Liver Disease	S-adenosyl-L-methionine
Oncology Treatments	Selenium
Eye Related Macular Disease	Beta-carotene and alpha-tocopherol
Pregnancy and Pre-eclampsia	Vitamin C and vitamin E supplements
Cardiovascular Risk Profile	Dietary antioxidants
Neonatal Growth Under Parenteral Nutrition (PN)	Cysteine, cystine or its precursor *N*-acetylcysteine
Melatonin and Cognitive Impairment or dementia	Melatonin
Alzheimer Disease	Vitamins C or E
Parkinson Disease	Tocopherol, CoQ10, and glutathione
Cancer	Lipid-soluble antioxidant vitamins
Asthma	Vitamin C, manganese etc
Cardiovascular Diseases	Vitamins C and E
Ischemia-Reperfusion Injury	Vitamin C
Chronic Obstructive Pulmonary Disease (COPD)	Polyphenol-rich pomegranate juice (PJ)
Pancreatitis	Selenium, L-methionine, and vitamins C and E
Rheumatoid Arthritis	Vitamins A, C, E or selenium or their combination
Kidney Diseases	Vitamin E
Liver Diseases	Antioxidant therapy
Diabetes Type I and II	Probucol and statins

CHAPTER 2: Materials and methods

2.1 Chemical characterization

2.1.1 Carqueja

The Carqueja used in this work was the *Pterospartum tridentatum* species. The parts from Carqueja that were used were flowers, as supplied by a natural products store (**Figure 12**). Each package contains 50 g of Carqueja flowers from plants growing in Portugal. The batches used are given in Appendix A.

Figure 12 Carqueja (*Pterospartum tridentatum*) used in extractions.

2.1.2 Waltheria *indica*

The parts of *Waltheria indica* used in this work were leaves, as supplied by Merck (Darmstadt, Germany), as shown in **Figure 13**.

Figure 13 *Waltheria indica* used in extractions.

2.1.3 Solvents, Reagents and Standards

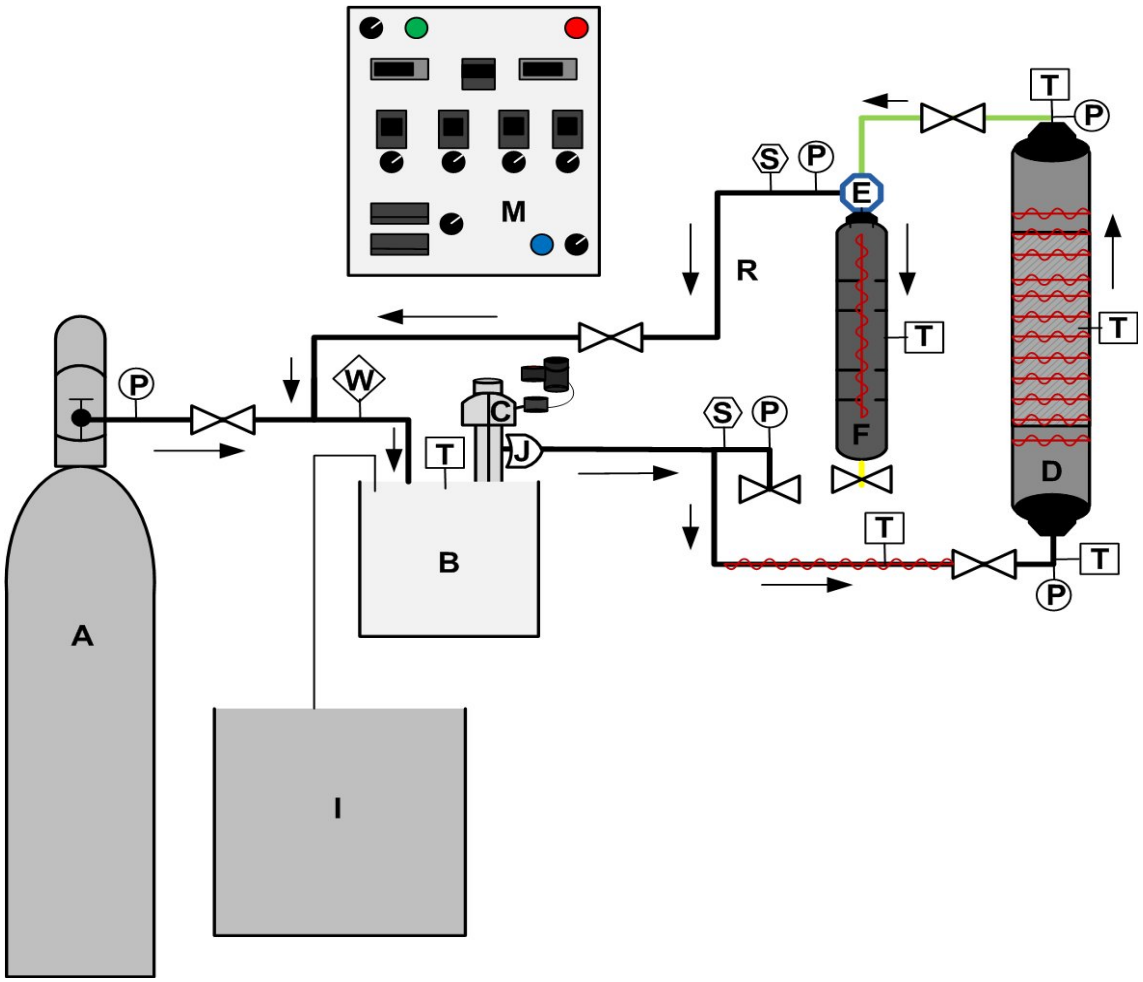
Table 9 Solvents, reagents and standards used.

Material	Supplier
2,2-diphenyl-1-picrylhydrazyl, 95%	Sigma-Aldrich
Acetone, 99.5%	Labchem
Acetyl Chloride, 98%	Fluka
Carbon Dioxide, 99.93%	Air Liquide
Dichloromethane, 99.8%	Sigma-Aldrich
Ethanol	Carlo-Erba
Ethyl Acetate	Carlo-Erba
Folin-Ciocalteu Reagent	Sigma-Aldrich
Gallic Acid, 98%	Sigma-Aldrich
Linoleic Acid, 99%	SAFC
Methanol 99.8%	Sigma-Aldrich
***N*-hexane, 96%**	Carlo-Erba
Sodium Carbonate, 99.5%	Sigma-Aldrich
Sulfuric Acid, 96%	Carlo erba
Trichloroacetic Acid, 99.5%	Scharlau

2.2 Extractions methods

2.2.1 $ScCO_2$

The SFE experiments were performed in the extraction apparatus shown in **Figure 16**, and shown schematically in **Figure 14**. The cylinder (**A**) releases CO_2 (the flow direction of which is represented by arrows). CO_2 is directed to a cold bath (**B**) filled with water and ethylene glycol acting as antifreeze, cooled by the cryostat (**I**) (Julabo F25), where it is cooled and liquified.



A – CO_2 Cylinder
B – Cold Bath
C – Liquid Pump
D – Extractor
E – BPR
F – Separator
I – Cryostat

J – Check Valve
M – Master Control Unit
P – Manometer
R – CO_2 Recycling Circuit
S – Safety Valve
T – Thermocouple
W – Flow Rate Indicator

– Valve
– Heating Jacket
– CO_2
– CO_2 + Oil
– Oil

Figure 14 Schematic diagram of the supercritical CO_2 extraction apparatus.

The liquid CO_2 is pumped by a pneumatic metering pump (**C**) (Williams V Series). It passes through a check valve (**J**) and is heated by a heating jacket connected to a thermocouple (**T**), to a set temperature between 40°C and 60°C, depending on the conditions desired. With increased temperature and pressure, CO_2 becomes a SCF.

The extractor (**D**) (HiP TOC 7-20-G Extractor), with an inner diameter of 2,5 cm and a length of 51,5 cm (Figure 15), is previously filled with the solid matrix, placed between cotton plugs.

Figure 15 HiP TOC 7-20-G Extractor used.

The Back Pressure Regulator, or BPR (**E**), ensures that pressure is maintained at the desired value – up to 500 bar – upstream, in the extractor (**D**).

The mixture exiting the extractor enters a separation vessel where its pressure returns to approximately 60 bar, which is the pressure of the CO_2 cylinder. With the drop in pressure and temperature, CO_2 turns into a gas and releases previously solubilized solutes/oil. The oil accumulates at the bottom of the separator (**F**) from where it can be easily recovered, by opening the valve below this vessel. Some CO_2 is lost in this procedure. However, most of the CO_2 goes to the recycling circuit (**R**), returning to the pre-cryostat section.

The flow rate of CO_2 is measured by a high-pressure flowmeter (RHEONIK 15 RHM 007 GTM) (**W**) and controlled by handling the pump's controls and slight adjustments of the BPR (**E**).

There are two safety valves (**S**), one after the pump and another after the BPR (**E**). Some of the pressure indicators are connected to a digital display in a master control unit (**M**) that, in case the pressure reaches a higher value than the one programmed, automatically shuts down the pump, for safety purposes. It is also to this master control unit (**M**) that the thermocouples (**T**) are connected, allowing almost all process control.

The extracts were collected in falcon tubes and weighed. The falcon tubes were wrapped in silver paper, sealed in a plastic bag and stored in the freezer. Subsequently, the yield of extraction was calculated, according to **Equation 1**:

Equation 1

$$Yield\ (\%) = \frac{Mass\ of\ extract\ (g)}{Mass\ of\ plant\ material\ (g)} \times 100$$

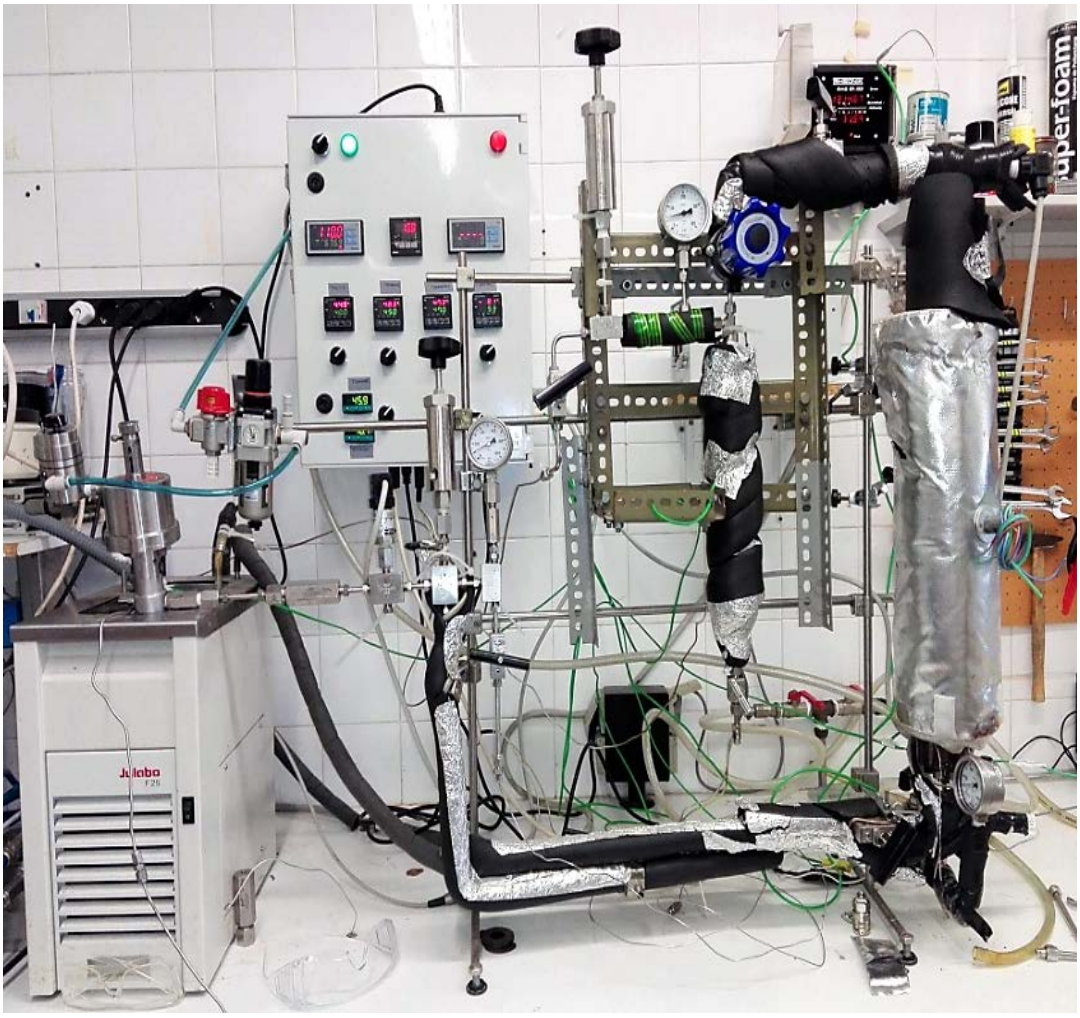
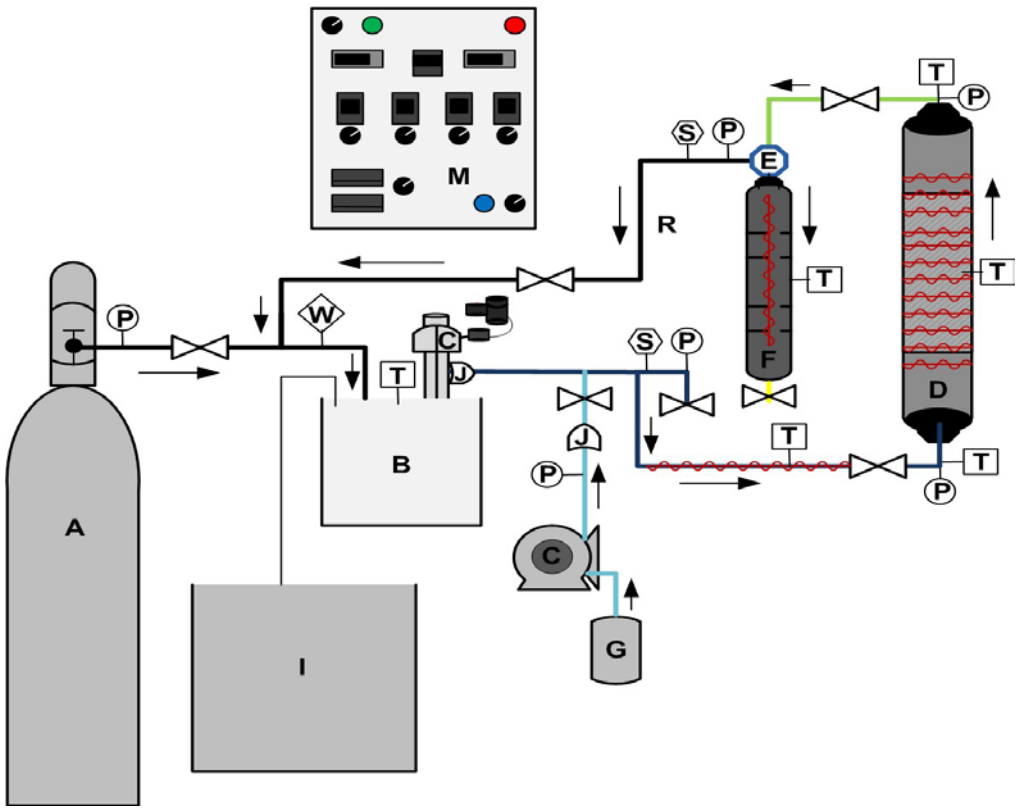


Figure 16 Image of the $scCO_2$ extraction apparatus.

2.2.2 ScCO_2 with co-solvent

The SFE experiments using a co-solvent were performed with the configuration of the extraction apparatus shown schematically in **Figure 17**. It is similar to the configuration shown in **Figure 14**, but with an extra section. In this work the co-solvent used was ethanol. Ethanol was pumped from its container (G) by means of a liquid pump (C), (LDC Minipump, model NSI-33R), and mixed with CO_2. The amount of ethanol used was determined by weighing the ethanol container at the start, along and at the end of the assay.



A – CO_2 Cylinder
B – Cold Bath
C – Liquid Pump
D – Extractor
E – BPR
F – Separator
G – Container of ethanol
I – Cryostat

J – Check Valve
M – Master Control Unit
P – Manometer
R – CO_2 Recycling Circuit
S – Safety Valve
T – Thermocouple
W – Flow Rate Indicator

– Valve
– Heating Jacket
– CO_2
– Ethanol
– CO_2 + Ethanol
– CO_2 + Oil
– Oil

Figure 17 Schematic diagram of the extraction apparatus with supercritical CO_2 using ethanol as co-solvent.

The extracts collected by opening the valve below the separator (F) were now a mixture of oil, ethanol and CO_2. Again, most CO_2 was recycled (R) back to the pre-cryostat section. The majority of the ethanol used was collected with the extracts (**Figure 18**). It was afterwards removed from the extracts using a line of gaseous nitrogen. Then, the same weighing and storage process described for pure scCO_2 was used.

Figure 18 Sample collection.

2.2.3 Soxhlet

The Soxhlet extraction process is a traditional experimental procedure used to obtain essential oils [4]. Therefore, Carqueja and Waltheria were submitted to this extraction method with the purpose of characterizing the samples and to compare with the composition of the extracts obtained by SFE.

In the process of extraction by Soxhlet, one should first choose the most suitable solvent for the sample, taking into account its polarity. The solvents used were *n*-hexane and ethanol, a nonpolar and a polar solvent, respectively. The chosen solvent was placed in a round bottom flask (250 mL or 500 mL). It was heated with a heating blanket to its boiling point, after which the reflux began. Before starting the extraction, a bag of filter paper was filled with the plant material and closed and placed in the chamber of the Soxhlet (50 mL or 250 mL). The solvent vapor passes through a distillation arm and when it touches the condenser, due to the temperature difference, it returns to the liquid state and fills the chamber where the previously mentioned bag is located. The hot solvent extracts the compounds from the material. Slowly, the chamber is filled with the hot solvent and with the compounds extracted. When the chamber is full, it is emptied by the siphon **(Figure 19).** The solvent returns to the round-bottom flask, completing the extraction cycle.

This cycle was repeated several times, for several hours. The solvents were subsequently removed from the extract, collected through a rotary evaporator (Nahita). After drying, the extracts were weighed and stored properly.

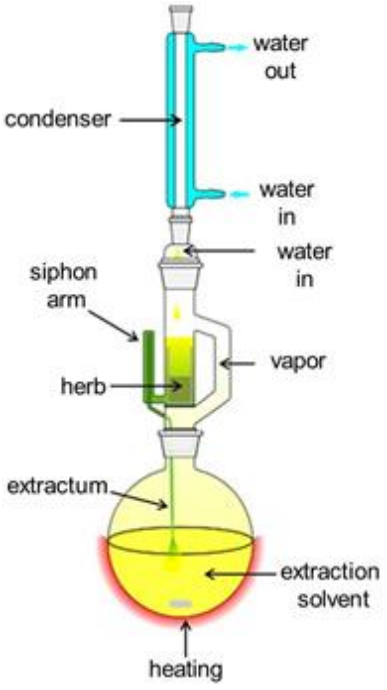


Figure 19 Laboratory size Soxhlet apparatus [74].

2.2.4 Infusion

The infusion preparation method used consisted in taking 200 mL of distilled water (pH = 6) to its normal boiling point (100 °C), and then adding 2 g of plant material, removing the heat source, and keeping the vessel with the infusion closed for 5 min (Figure **20**). The resulting solution was filtered under reduced pressure. To facilitate storage, the infusion was frozen with liquid nitrogen and freeze-dried later.

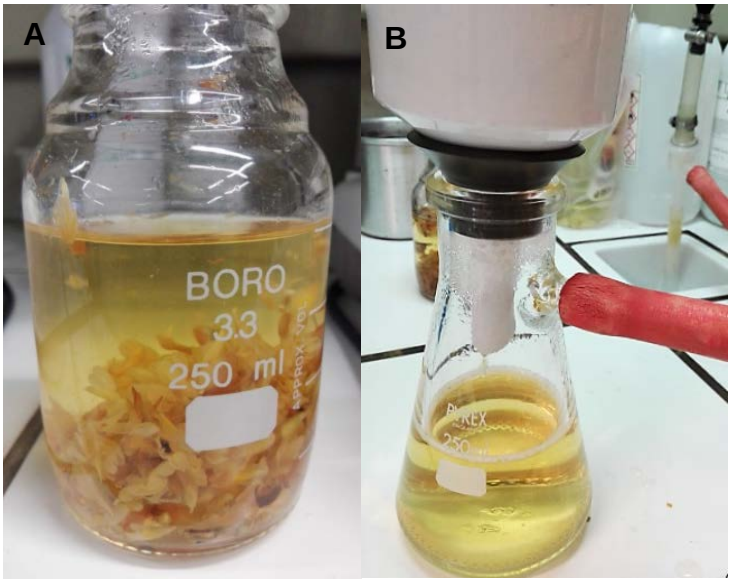


Figure 20 (A) Carqueja infusion, and (B) infusion after it was filtered under reduced pressure.

2.3 Analytical Processes

2.3.1 Thin layer chromatography (TLC) analysis of extracts

The P. *tridentatum* extracts obtained with $scCO_2$ and through the Soxhlet with ethanol and Soxhlet with *n*-hexane extractions were analysed by TLC. Silica plates (Macherey-Nagel xtra SilG/UV254, Ref. 818333) were used.

Assays were first done to assess the best eluent and universal stains. Several elution solvents were tested, namely *n-hexane*, ethyl acetate, dichloromethane, ethanol and methanol, as well as mixtures of these solvents. The chromatography standards used to identify substances in the Carqueja extracts were linoleic acid (for free fatty acids), olive oil (for triglycerides) and *(+)*-ledene ((1S,2R,4R,11R)-3,3,7,11-tetramethyltricyclo[6.3.0.02.4]undec-7-ene) and trans-caryophyllene (trans-(1R,9S)-8-methylene-4,11,11-trimethylbicyclo[7.2.0] undec-4-ene) (for terpenes). In the case of the ethanol extract, the following standards were used to look for the presence of phenolic compounds: pyrogallol (benzene-1,2,3-triol), gallic acid (3,4,5-trihydroxybenzoic acid), and borneol (endo-(1S)-1,7,7-trimethylbicyclo[2.2.1] heptan-2-ol).

Beforehand, the silica plate was prepared with the respective extracts of Carqueja and the chromatography standards. The chosen eluent was placed in a closed elution chamber, and then the plate was inserted in the chamber. It was kept there for a few minutes, until the solvent reached the top of the plate.

The universal stains tested were potassium permanganate (1,5 g $KMnO_4$, 10 g of K_2CO_3, 1,25 mL 10% methanol, 200 mL H_2O), phosphomolybdic acid (PMA, 20% ethanol, **Figure 21**), sulphuric acid (sulphuric acid diluted in methanol, 1:1) and 3,4-dinitrophenylhydrazine.

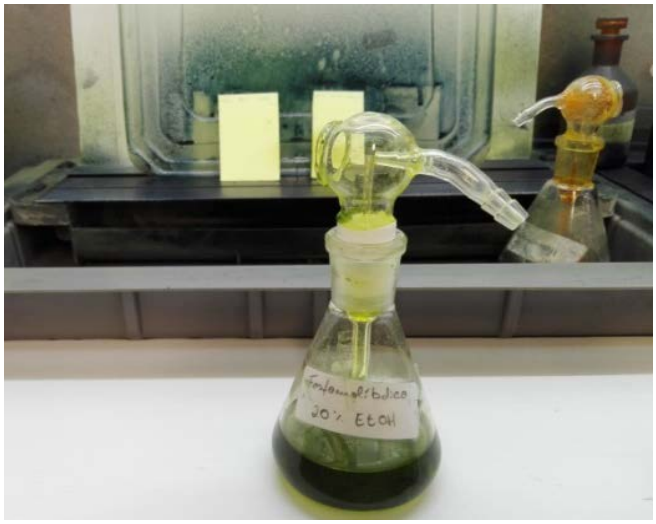


Figure 21 The universal stain phosphomolybdic acid.

2.3.2 Total phenolic content - Folin-Ciocalteu method

In the colorimetric Folin-Ciocalteu method the oxidation of phenols by a molybdotungstate reagent produces a colored product with a maximum wavelength (λ_{max}) at 745-750 nm. Through the action of the phenolic compounds of the samples, the Folin-Ciocalteau reagent is reduced, forming a blue complex (**Figure 22**). This method has been used over the years, as a measure of quantification of total phenolics in natural products since it is a simple and precise method. However, the reaction is slow at acid pH and lacks specificity [75].

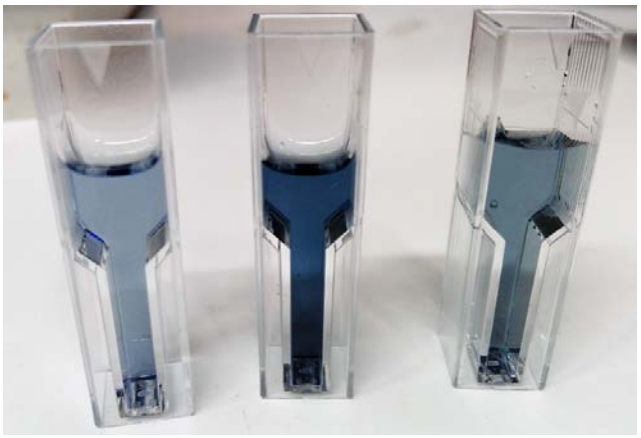


Figure 22 Reduction by phenolic compounds in the extracts from P. *tridentatum* flowers, forming a blue complex.

The Folin-Ciocalteu method was used with the objective of quantifying the phenolic compounds presented in extracts of P. *tridentatum* and W. *indica*, which may be responsible for their antioxidant capacity. The method was used for three types of extracts: scCO_2/ethanol, Soxhlet/ ethanol and infusion/water.

To perform the Folin-Ciocalteu method, gallic acid was used as the reference compound. A gallic acid standard solution was prepared from a stock solution of 5 g/L, with concentrations of 10, 25, 50, 100, 150, 250, 500 mg/L in distilled water. The gallic acid calibration curve is in Appendix B.

First the samples were pretreated to perform a protein precipitation step. The extract was dissolved in an appropriate solvent (5 mg/mL). To 800 µL of extract solution was added 120 µL of trichloroacetic acid solution (100 g/ 100 mL) (TCA). The solution was well stirred and stored for 5 min at -20 °C, followed by 15 min at 4 °C. Afterwards, the solution was centrifuged. The precipitate was discarded, and 20 µL of the recovered supernatant were mixed with 1.58 mL of distilled water and 100 µL of Folin-Ciocalteu reagent. Three replicates of each extract were made. They were well stirred and incubated for 5 min. After this time period, it was added 300 µL of sodium carbonate solution, followed by incubation at 40°C for 30 min, in an Accu BlockTM

Digital Dry Bath. The absorbance of the samples was then measured at 750 nm with a DU® 800 Spectrophotometer from Beckman Coulter, Brea, USA [76].

For the calculation of total phenolic compounds (TPC), **Equation 2** was used. Results were expressed as mg of gallic acid equivalent per g extract.

Equation 2

$$TPC\ \left(\frac{\mathrm{mg}_{Gallic\ acid\ equiv}}{\mathrm{g}_{extract}}\right) = \frac{C\ (\frac{mg_{Gallic\ acid\ equiv}}{L})}{g_{extract}} \times V\ (L)$$

2.3.3 Antioxidant activity. DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The antioxidant activity of the extracts was evaluated with the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. The DPPH radical has a deep purple color and is one of the few stable organic nitrogen radicals (**Figure 23**). The free radical DPPH test is based on the measurement of the antioxidant reduction capacity, which can be evaluated by measuring the DPPH color loss after reacting with solutions prepared from extracts [75]. The color gradually changes from deep purple to yellow, as shown in **Figure 23**. This change in color is monitored by spectrophotometry at 517 nm.

Figure 23 Color change from the original purple color of the DPPH radical to yellow, upon reaction with the solutions prepared with the extracts.

The antioxidant activity was evaluated in both extracts, from P. *tridentatum* and from W. *indica,* for all the types of extractions made: $scCO_2$/ethanol, Soxhlet/ethanol, infusion/water, and Soxhlet/*n*-hexane.

Before starting the free radical DPPH test, the stock solution was previously prepared by dissolving 24 mg of DPPH in 100 mL of methanol. It is stored at -20°C for a minimum of 2 hours. The absorbance measured is monitored spectrophotometrically at 517 nm until absorbance reaches a value near 1 (not more than 1.1) [76].

The extracts were dissolved in a chosen solvent, in different concentrations ranging between 300 and 10000 mg/L. Three replicates for each extract were made. As light can interfere with the radicals, the reaction should be performed in the dark and with the use of amber vials. In these vials were placed 4 mL of the stock solution that were mixed with 150 µL of the solutions of extract. The mixtures were stored for 40 min. After this time, the samples were well mixed and the absorbance measured at 517 nm.

The inhibition of the free radical DPPH, by each sample, was calculated by **Equation 3**:

Equation 3

$$\%\ of\ inhibition = \frac{A_{DPPH} - A_{Sample}}{A_{DPPH}}$$

where A_{DPPH} is the absorbance of the blank (DPPH solution) and A_{sample} is the absorbance of the sample with extract (DPPH solution + solution of extract). The half maximum effective concentration (EC_{50}) of each sample was calculated from the inhibition curves to evaluate the antioxidant activity of the extracts. [76] For easy understanding and comparing with literature, the results are also given in mg of extract per mg of DPPH.

CHAPTER 3: Results and discussion

3.1 Extraction

3.1.1 $ScCO_2$ efficiency process

SFE extraction experiments were carried out in the extraction apparatus shown schematically in **Figure 14**. Several assays with Carqueja were performed (**Table 10**). The amount of Carqueja used ranged between 42 and 48.5 g. To evaluate the influence of temperature and pressure, the operating conditions were varied between 40 and 60 °C and between 350 and 500 bar, with a flow rate ranging between 6 and 15 g min^{-1}.

Table 10 Parameters and yields of the extractions made with $scCO_2$, for Carqueja and W. *indica*.

Plant	Mass of plant (g)	Co-solvent	Solvent flow rate (g/min)	T (^oC)	P (bar)	Mass of extract (g)	Yield (%)
Carqueja	42.00	-	15.00	40	350	0.270	0.64
Carqueja	48.00	-	15.00	40	500	0.614	1.28
Carqueja	48.50	-	10.05	60	500	0.045	0.10
Carqueja	48.00	-	11.55	60	500	0.112	0.23
Carqueja	46.90	Ethanol (5 wt.%)	20.89	40	300	0.575	1.23
Carqueja	48.00	Ethanol (9 wt.%)	18.63	40	300	1.296	2.70
Carqueja	47.00	Ethanol (14 wt.%)	20.23	40	300	2.050	4.36
W. *indica*	38.85	Ethanol (5 wt.%)	19.63	40	300	0.785	2.02
W. *indica*	38.00	Ethanol (5 wt.%)	19.58	40	300	0.723	1.90
W. *indica*	38.00	Ethanol (10 wt.%)	25.29	40	300	1.188	3.13
W. *indica*	38.00	Water	15.09	40	300	0.065	0.17

In the first assay, the extracts were collected every 10 min, for 3 h. In all the remaining experiments, extracts were collected until a maximum of 2 h of extraction, and normally every 10 or 20 min. Temperature, pressure, and mass flow rate readings were taken at the same time intervals, and so were the Carqueja extract samples collected in falcon tubes. This procedure allowed to quantify the extracted oil, by weighing, and later to obtain the curves of extraction (**Figure 24**, **Figure 25**).

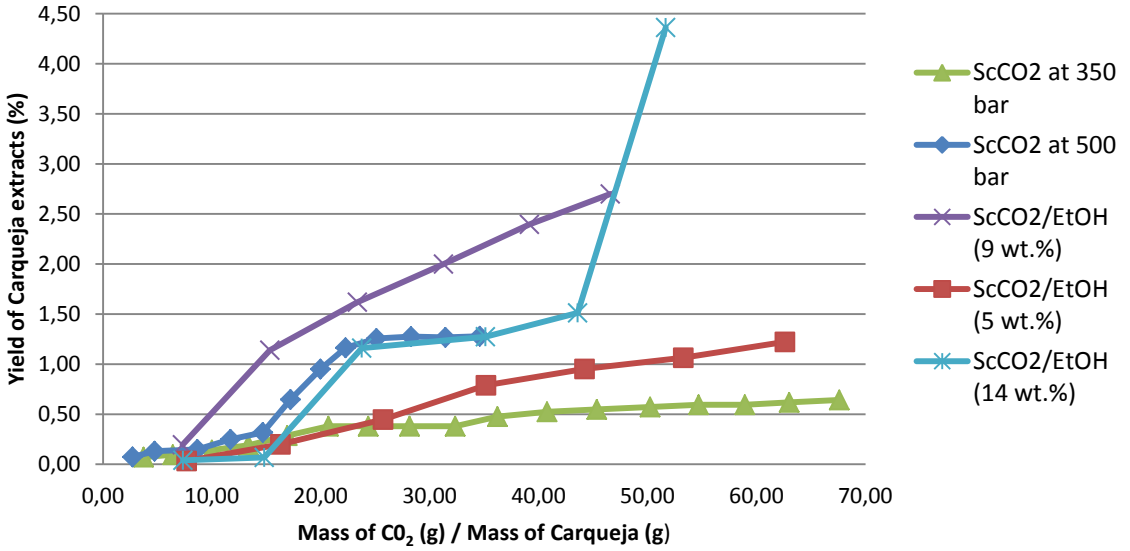


Figure 24 Extraction curves for Carqueja at 40 °C.

As expected, and as seen in **Table 10**, at a constant temperature of 40 °C, an increase in pressure from 350 to 500 bar led to higher oil yields during the assays. Increasing the extraction pressure at constant temperature results in higher densities of $scCO_2$, which leads to an increase in the Carqueja oil solubility. The results show that at 500 bar, $scCO_2$ extracted the oil that was available fast, until the yield of extraction stabilized. At 350 bar, the lower solubility of the oil in $scCO_2$ required much larger amounts of solvent to remove the oil available in the plant material. It is to be expected that the same yield of extraction would be reached if the assay had taken much longer, given the slope of the curve at 350 bar.

Both pressure and temperature have an influence on the extraction yield. At constant pressure, an increase in temperature lowers the density of $scCO_2$, which also lowers solute solubility, unless it is counterbalanced by an increase in vapor pressure of the solutes. The density of CO_2 at 40 ºC varies between 0.91 and 0.99 g cm^{-3}, in the range 300 - 500 bar, whereas at 60 ºC it varies between 0.83 and 0.93 g cm^{-3}, in the same pressure range [77].These differences cannot explain yields at 60 ºC down to half of those at 40 ºC, at constant pressure. There was some difficulty in keeping operating conditions stable at 60 ºC, which may be behind the results obtained.

Additional extraction runs were performed with ethanol as co-solvent. These SFE experiments were carried out in the extraction apparatus shown schematically in **Figure 17**. The amount of Carqueja material ranged between 47 and 48 g, and in the case of W. *indica,* between 38 and 39 g. The assays were run at 40 °C and 300 bar for both materials, although with a different CO_2 flow rate and co-solvent mass flow rates. In extractions with Carqueja, the CO_2 flow rate ranged from 19 to 21 g min^{-1} and the co-solvent was added at 5, 9 and 14 wt.%. In extractions

with W. *indica,* the CO_2 flow rate ranged from 20 to 25 g min^{-1}, and the co-solvent was added at 5 and 10 wt.% (**Table 10**).

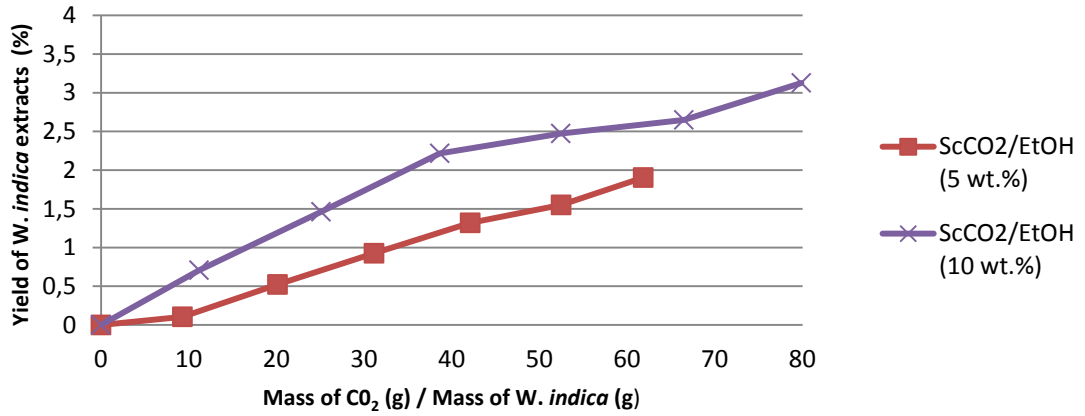


Figure 25 Extraction curves of W. *indica* with a constant pressure of 300 bar and temperature of 40 °C.

As shown in **Table 10**, the addition of ethanol as co-solvent had a significant influence on the extraction yield. As a matter of fact, ethanol is the solvent that increases the extraction of polyphenols and specific flavonoids the most [78], which are the main components present in the plants under study. In the case of Carqueja, a small addition of co-solvent (5 wt.%) led to similar yields as the $scCO_2$ extraction at a higher pressure (500 bar). However, an important factor to consider is the energy consumption in the process, which means that higher pressures require higher power consumption to compress the solvent.

Comparing the three $scCO_2$ extractions with co-solvent for Carqueja, it is evident that by increasing the mass flow rates of the co-solvent, so that the amount of ethanol in CO_2 was increased from 5 to 9, and then to 14 wt.%, the yield also increased, from 1.23, to 2.70, and then to 4.36%, respectively. The extraction curve for the latter condition reflects a problem that arose in the experimental assay, causing difficulties in collecting the extract, which were overcome before the end of the assay.

As with Carqueja, the increase in ethanol flow rates also resulted in increased extraction yields for W. *indica*, but this increase in yield was less pronounced than in the case of Carqueja.

The higher yields of extraction when ethanol was used as co-solvent can be explained by the increase in polarity of the solvent mixture, which becomes capable of dissolving other compounds that CO_2 alone cannot extract. CO_2 has a solvent strength similar to that of hexane, as shown in **Figure 26** [79], where solvent strength is represented by data for two different solvatochromic dyes. The authors of this work have shown that when a small amount of

methanol is added, the solvent strength of the CO_2/methanol mixture increases substancially relative to pure CO_2, due to the presence of a substance (methanol) capable of establishing hydrogen bonds. However, solvent strength increases non-linearly with the increase in methanol concentration. This is attributed to the formation of methanol clusters as methanol is first added to CO_2. Polar solutes interact primarily with these clusters, and are solubilized to a greater extent, while nonpolar solutes continue to interact primarily with CO_2 molecules, and show no increase in solubility. It is reasonable to assume that the behavior of ethanol as co-solvent is similar to that of methanol, although less pronounced, due to its lower polarity relative to methanol.

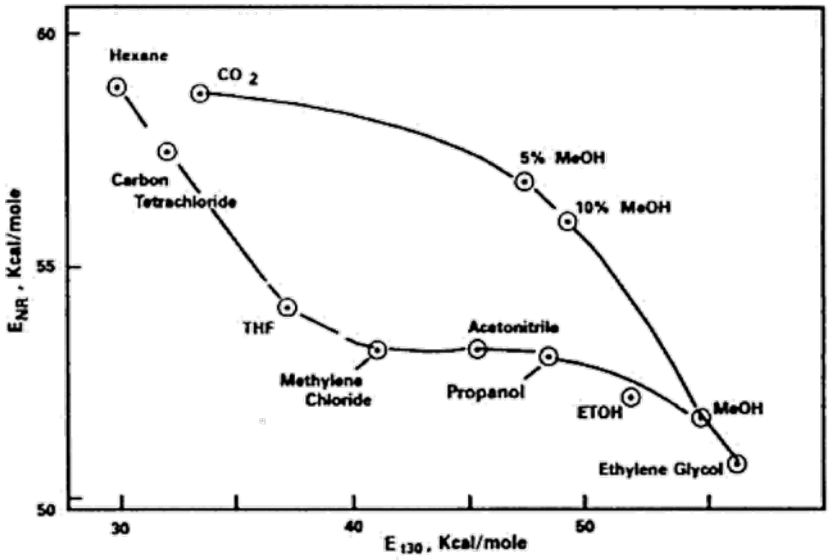


Figure 26 Relative solvent strengths of various solvents [79].

Additionally, an assay was carried out with W. *indica* sprinkled with water. This assay generated an extract that changed in color as the assay progressed, from a color similar to that of Soxhlet/ethanol extracts to that of infusion/water extracts. But the apparatus used, in its present configuration, did not allow the recirculation of $scCO_2$ containing high amounts of water. Therefore the first $scCO_2$ to contact the material absorbed water, but as the assay continued the material became drier, and there was no longer water available to behave as co-solvent. This can explain the change in color of the extract obtained. The yield was very low, as shown in **Table 10**: 0.2 wt.%.

For the two species studied, no reports of SFE assays were found in the literature with which to compare data.

3.1.2 Soxhlet efficiency process

The Soxhlet extraction was made for comparison with SFE. The extraction was performed with two differents solvents, namely *n*-hexane and ethanol. The extractions with *n*-hexane were carried out at its normal boiling point (68.7 °C). Two assays were made, one of them with 24 g of Carqueja and 300 mL of solvent, which ran for 4 h, and the other was made with 25 g of Carqueja and 300 mL of solvent, which ran for 6 h. *N*-hexane was subsequently removed from the collected extract with a rotary evaporator. The results obtained are shown in **Table 11**.

The extractions with ethanol were carried out at its normal boiling point (78.4 °C). Approximately 25 g of Carqueja and 300 mL of solvent were used for each assay. With these conditions, three assays were made. One of them ran for 4 h and the other two ran for 6 h. These assays were also made for W. *indica*, with 5 g of material and 80 mL of solvent. Two replicas were made, both of which ran for 4 h. The ethanol was subsequently removed from the collected extract with a rotary evaporator.

In addition, extractions with ethanol were peformed for Carqueja left in the extractor after the extration with $scCO_2$ (500 bar), and after the extraction with $scCO_2$/Ethanol (9 wt.%). Both of these Soxhlet assays ran for 4 h, and were performed using 6.5 g of material and either 75 mL or 80 mL of solvent. Likewise, one extraction was made using W. *indica* left in the extractor after the extration with $scCO_2$/Ethanol. This Soxhlet/ethanol assay ran for 4 h, and was carried out with 5 g of material and 80 mL of solvent.

The results obtained for the different methods of extraction are shown in **Table 11**. When using *n*-hexane, the extraction yield obtained for Carqueja was very similar in the two assays performed, which suggests that the compounds soluble in hexane were extracted after running the assay for 4 h.

Noticeably the extraction yield for Carqueja was much higher when using ethanol than when using hexane. Ethanol is a polar solvent, whereas hexane is a non-polar solvent. The results obtained indicate that there are many more polar solutes in Carqueja, soluble in ethanol, than non-polar ones, soluble in *n*-hexane.

In the case of the Soxhlet/ethanol experiments, both the time and number of cycles were recorded. The yield of extraction increased as the number of cycles increased from 2 to 17. The cycles correspond to the number of times the solvent is in contact with the material. So long as there are components to extract by a given solvent, it is expected to observe an increase in yield of extraction with the increase in the number of cycles. The differences between the number of cycles that occurred, when faced with the same solvent and time interval, can be justified by the difficulty in controlling the temperature.

One way to see if the number of cycles is sufficient is observing the change in color of the solvent that comes down from the siphon. Both in the case of Carqueja and of W. *indica*, at the

end of the assays with a larger number of cycles the solvent appeared to have the same color as pure solvent.

The highest extraction yield of Carqueja with the Soxhlet was 21.5 %. However, the result obtained for the Soxhlet/ethanol assays using Carqueja left in the extractor after extraction with $scCO_2$ (500 bar) suggests a higher yield of extraction than obtained for Carqueja as supplied commercially. This may be explained by the fact that the compounds being extracted by ethanol are the same, but now the plant material is comparatively a little richer in those compounds, having lost less polar substances when it was extracted with pure $scCO_2$. This effect is not apparent in the case of Carqueja left in the extractor after extraction with $scCO_2$/Ethanol (9 wt.%). This is probably because the presence of ethanol as co-solvent gave the solvent mixture the capacity to extract compounds with a polarity lower than that of ethanol, but higher than that of pure $scCO_2$. These trends should be confirmed with additional experiments.

In the case of W. *indica*, previous extraction with $scCO_2$/Ethanol (10 wt.%) seems to lead to a slightly higher yield of extraction than obtained for the material as supplied. But as before, additional experiments would be needed to confirm this trend.

Table 11 Experimental parameters and extraction yields when using the Soxhlet method, for Carqueja and W. *indica*.

Plant	Mass of plant (g)	Solvent	Volume of solvent (mL)	Time (h)	T (^oC)	Mass of extract (g)	Yield (%)
Carqueja	23.93	*n*-Hexane	300	4	68	0.38	1.6
Carqueja	24.53	*n*-Hexane	300	6	68	0.41	1.7
Carqueja	24.70	Ethanol	300	4 (2 cycles)	78	2.97	12.0
Carqueja	24.95	Ethanol	300	6 (17 cycles)	78	4.83	19.4
Carqueja	24.70	Ethanol	300	6 (17 cycles)	78	5.30	21.5
***R_2 Carqueja**	6.50	Ethanol	75	4 (38 cycles)	78	1.39	21.4
***R_1 Carqueja**	6.50	Ethanol	80	4 (46 cycles)	78	1.54	23.7
W. *indica*	5.00	Ethanol	80	4 (8 cycles)	78	0.77	15.4
W. *indica*	5.00	Ethanol	80	4 (24 cycles)	78	1.12	22.4
R_1 W. *indica	5.00	Ethanol	80	4 (22 cycles)	78	1.20	23.9

*R_1 Carqueja = Carqueja left in extractor after the extration with $scCO_2$ (500 bar); R_2 Carqueja = Carqueja left in extractor after the extration with $scCO_2$/EtOH (9 wt.%); R_1 W. *indica* = W. *indica* left in extractor after the extration with $scCO_2$/EtOH (10 wt.%).

3.1.3 Infusion efficiency process

All the infusion/water experiments, both for Carqueja and for W. *indica* material, were made as described in chapter 2.2.4. The highest yield obtained in the infusion was 25.0%, obtained with W. *indica* material. For Carqueja material, the highest yield was 22.5% (**Table 12**).

When analyzing the results obtained for the plant materials previously submitted to extraction with $scCO_2$/Ethanol, it seems that the trend in the results is for lower yields of extraction compared to the plant materials as supplied. This can be seen by comparing 21.8 and 22.5% with 18.5 and 20.0 % for Carqueja, and by comparing 25.0% with 20.5% in the case of W. *indica*. This trend is opposite to that observed with Carqueja, indicating that cCO_2, with or without cosolvent, was able to extract some compounds that were also extracted through infusion/water. This effect is more pronounced in the case of Carqueja, especially when analyzing results for plant metarial previulsly extracted with $scCO_2$ alone.

These trends should be confirmed with additional experiments. Understanding these observations would require knowledge of the type of compounds present in the materials.

Table 12 Experimental parameters and extraction yields when using the Soxhlet method, for Carqueja and W. *indica*.

Plant	Volume of solvent (mL)	Mass of extract (g)	Yield (%)
Carqueja	215	0.35	17.5
Carqueja	200	0.44	21.8
Carqueja	200	0.45	22.5
***R_1 Carqueja**	200	0.37	18.5
***R_2 Carqueja**	200	0.40	20.0
W. *indica*	200	0.50	25.0
R_1 W. *indica	200	0.41	20.5

*R_1 Carqueja = Carqueja left in extractor after the extration with $scCO_2$ (500 bar); R_2 Carqueja = Carqueja left in extractor after the extration with $scCO_2$/EtOH (9 wt.%); R_1 W. *indica* = W. *indica* left in extractor after the extration with $scCO_2$/EtOH (10 wt.%).

3.1.4 Carqueja overall efficiency process

All the Carqueja extracts exhibited a characteristic odor. They also exhibited a color that depended on the type of extraction used.

As it can be seen in **Figure 27,** the $scCO_2$ extracts were yellowish and the infusions had a strong yellow hue before drying. In the Soxhlet assay with *n*-hexane, a yellow hue could be seen at the beginning, which disappeared (**Figure 28**). However in the Soxhlet assays with ethanol, the hue was green from the start and got increasingly darker, having a more oil-like appearance (**Figure 29**). These distinctions are justified by the polarity difference between the solvents used and, consequently, of the different compounds extracted. This evidence shows that in addition to organic compounds, aromas and pigments have also been extracted.

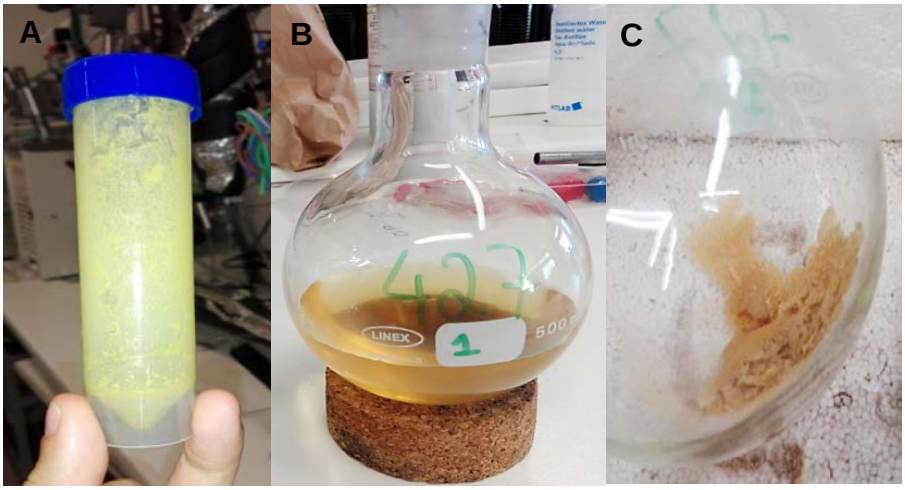


Figure 27 Image of (A) $scCO_2$ extract of Carqueja. Also images of infusion (B) and (C) dried extract.

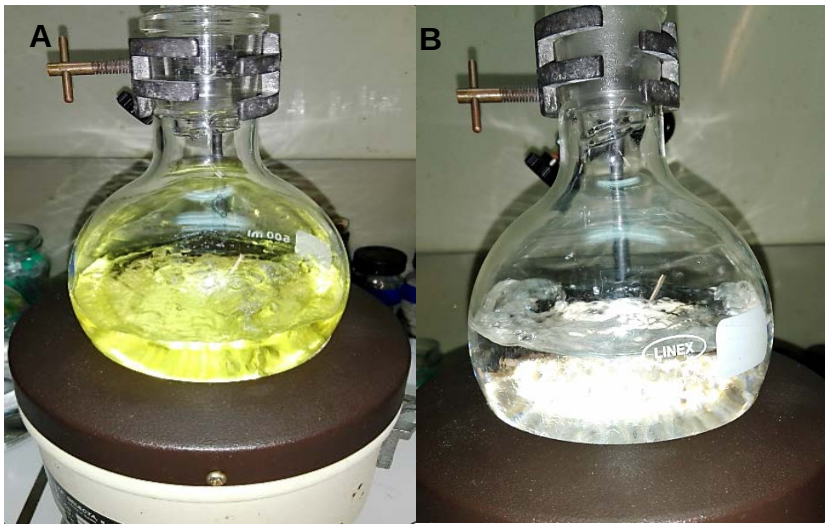


Figure 28 Images of Soxhlet extraction of Carqueja with *n*-hexane (A) at the beginning and (B) end of the assay.

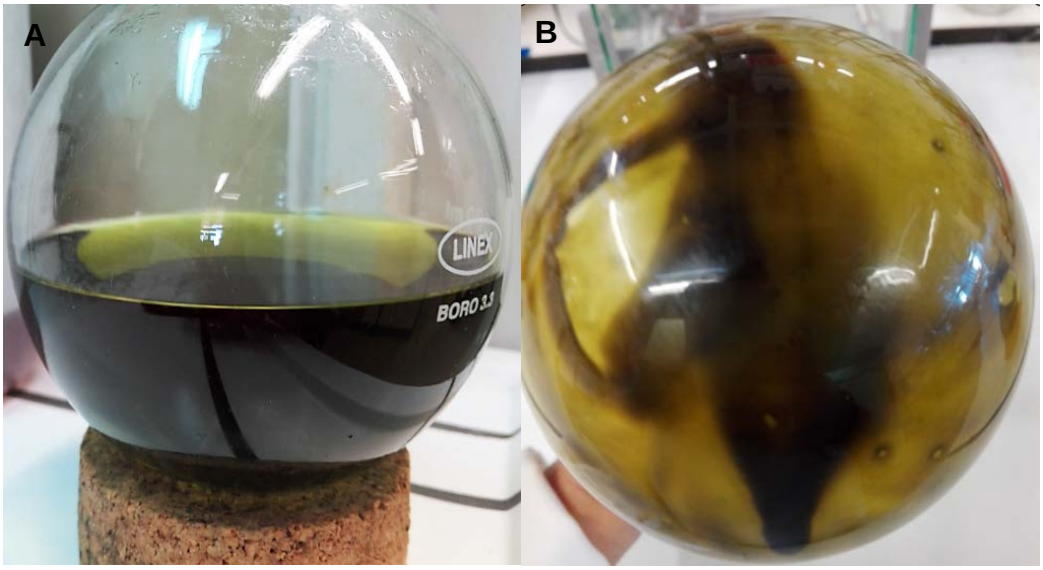


Figure 29 Images of Soxhlet extraction of Carqueja with ethanol (A) before and (B) after solvent removal.

In **Table 13,** data for all the extractions made for Carqueja are shown. All the assays made using non-polar or low polarity solvents, such as *n*-hexane, $scCO_2$ and $scCO_2$ with co-solvent show low extraction yields, which is to be expected considering that Carqueja presents polyphenols as major components. These polar compounds appear to be extracted in the processes that use polar solvents, such as ethanol and water, resulting in much higher yields of extraction.

Of all the assays performed, the one that presented the highest yield was the infusion, with 22.5%. This value falls within those given in the literature, as shown in **Table 14**, which gives conditions of extraction and yields reported by several authors for the P. *tridentatum* species. As described in the literature, these extractions could yield different results if other parts of the plant were used, if the location and height of the crop were different, and if there had been monitoring of the particle size of the material extracted.

In this work it was possible to reach a yield of 23.7% when performing a pre-extraction step of Carqueja material with $scCO_2$, followed by extraction with ethanol. This indicates that, even though the extractions with $scCO_2$ have low yields compared with extractions with other solvents, they recover compounds that ethanol alone is not able to extract, allowing some degree of fractionation of the plant material. The opposite was found when Carqueja previously extracted with $scCO_2$ was extracted with water, where results suggest that CO_2 and water essentially target different compounds, as revealed by the large differences in yields of extraction. However there is a small amount of compounds in Carqueja that both solvents can extract.

Table 13 Experimental parameters and extraction yields for the three types of extraction made when using Carqueja material.

Process	Mass of plant (g)	Solvent flow rate (g/min)	Volume of solvent (mL)	Time (h)	T (°C)	P (bar)	Mass of extract (g)	Yield (%)
$ScCO_2$	42.00	15.00	-	3	40	350	0.270	0.64
$ScCO_2$	48.00	15.00	-	2	40	500	0.614	1.28
$ScCO_2$/EtOH (5 wt.%)	46.90	20.89	-	2	40	300	0.575	1.23
$ScCO_2$/EtOH (9 wt.%)	48.00	18.63	-	2	40	300	1.296	2.70
$ScCO_2$/EtOH (14 wt.%)	47.00	20.23	-	2	40	300	2.050	4.36
Soxhlet/*n*-Hexane	23.93	-	300	4	68	-	0.38	1.6
Soxhlet/*n*-Hexane	24.53	-	300	6	68	-	0.41	1.7
Soxhlet/EtOH	24.70	-	300	4 (2 cycles)	78	-	2.97	12.0
Soxhlet/EtOH	24.95	-	300	6 (17 cycles)	78	-	4.83	19.4
Soxhlet/EtOH	24.70	-	300	6 (17 cycles)	78	-	5.30	21.5
Soxhlet/EtOH *R_2	6.50	-	75	4 (38 cycles)	78	-	1.39	21.4
Soxhlet/EtOH *R_1	6.50	-	80	4 (46 cycles)	78	-	1.54	23.7
Infusion	2	-	215	-	100	-	0.35	17.5
Infusion	2	-	200	-	100	-	0.44	21.8
Infusion	2	-	200	-	100	-	0.45	22.5
Infusion *R_1	2	-	200	-	100	-	0.37	18.5
Infusion *R_2	2	-	200	-	100	-	0.40	20.0

*R_1 = Carqueja left in extractor after the extration with $scCO_2$ (500 bar); R_2 = Carqueja left in extractor after the extration with $scCO_2$/EtOH (9 wt.%);

Table 14 Carqueja extraction conditions and yields reported in the literature.

Process	Local and period of collection	Parts and mass of plant (g)	Volume of solvent (mL)	Time (h)	Yield (%)	REF
EtOH extract	-	(10)	100	12.00	60.2 ± 0.225	[29]
EtOH extract (80%)	Pombal, Flowering	Flowers (25)	-	0.50	17.00 ± 1.13	[30]
H_2O extract	Pombal, Flowering	Flowers (25)	1000	0.25	14.91 ± 2.50	[30]
H_2O extract	Cinfães	Flowers (50)	1500	0.17	30	[28]
Soxhlet/H_2O	Gardunha, Flowering	Flowers (50)	250	2.00	19.4 ± 1.22	[27]
Soxhlet/H_2O	Gardunha, Flowering	Stems (50)	250	2.00	14.9 ± 0.83	[27]
Soxhlet/H_2O	Gardunha, Dormancy	Stems (50)	250	2.00	15.7 ± 1.37	[27]
Soxhlet/H_2O	Malcata, Flowering	Flowers (50)	250	2.00	14.8 ± 0.26	[27]
Soxhlet/H_2O	Malcata, Flowering	Stems (50)	250	2.00	11.3 ± 1.21	[27]
Soxhlet/H_2O	Malcata, Dormancy	Stems (50)	250	2.00	18.9 ± 1.31	[27]
Soxhlet/H_2O	Orvalho, Flowering	Flowers (50)	250	2.00	16.8 ± 1.56	[27]
Soxhlet/H_2O	Orvalho, Flowering	Stems (50)	250	2.00	12.8 ± 0.56	[27]
Soxhlet/H_2O	Orvalho, Dormancy	Stems (50)	250	2.00	15.8 ± 0.93	[27]
Soxhlet/MeOH	Serra Da Estrela	S&L (100)	1000	-	26.1 ± 1.7	[37]
Soxhlet/MeOH	Serra Da Estrela	Flowers (100)	1000	-	26.1 ± 1.7	[37]

3.1.5 W. *indica* overall efficiency process

All the W. *indica* extracts exhibited a characteristic odor and color. As seen in Figure **30** , both the extracts obtained with $scCO_2$/ethanol and Soxhlet/ethanol present a green hue, and the infusion presents a yellow hue. As in the case of the Carqueja, these differences in pigmentation are due to the differences in polarities of the solvents and the different compounds they are able to extract.

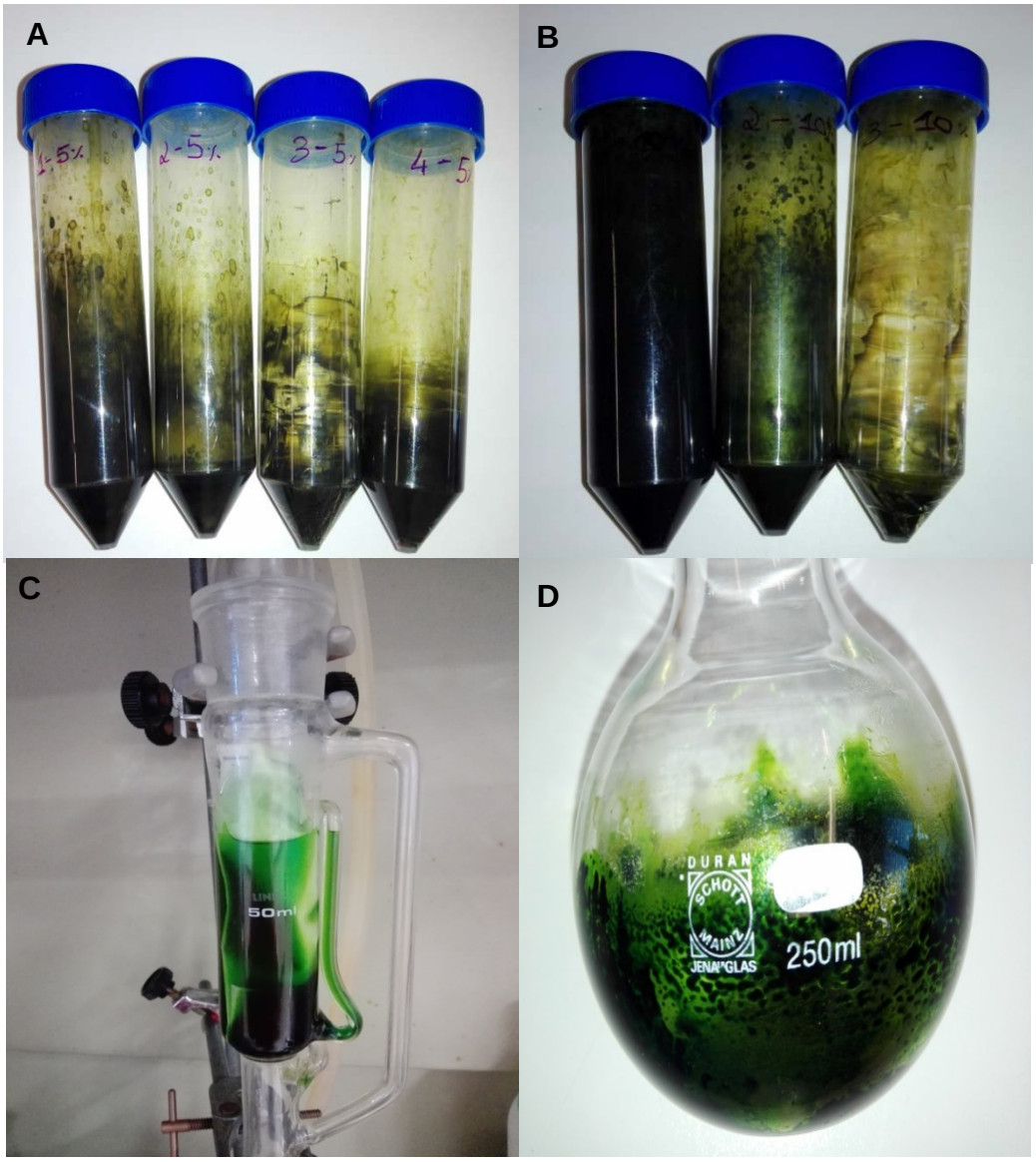


Figure 30 Images of extractions and extracts for W. *indica*. $ScCO_2$ extraction with (A) 5 wt.% of ethanol and (B) 10 wt.% of ethanol, sample chamber in Soxhlet extraction with ethanol (C) and (D) dried extract.

Of the various assays made with W. *indica,* shown in **Table 15**, the one that presented the highest extraction yield was the infusion (25.0%, **Figure 31**). This value is similar to the highest value referred in the literature, of 23.8% [77], as shown in **Table 16**. However, and as mentioned earlier, additional assays should have been performed for a more precise determination of extraction yields.

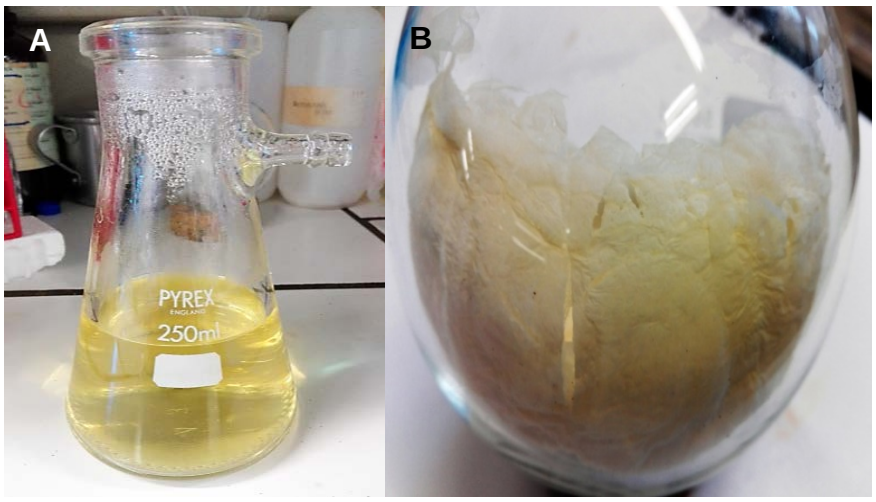


Figure 31 Photo of W. *indica* infusion (A) before and (B) after drying.

Taking into account that the regularly occurring chemical constituents of W. *indica* are flavonoids [57], it was expected to obtain higher yields of extraction when using water or ethanol as extraction solvents than when using $scCO_2$.

Indeed the $scCO_2$/ethanol assays gave much lower yields than those obtained with Soxhlet/ethanol. Similarly to Carqueja, it was possible to reach a higher yield of extraction using Soxhlet/ethanol after the plant material had been submitted to $scCO_2$ extraction, which again suggests that in this two-step process some degree of fractionation was achieved.

Also as observed with Carqueja, W. *indica* that remained in the extractor after extraction with $scCO_2$/Ethanol gave lower yields of extraction than the original material, which indicates the presence of a small amount of compounds in W. *indica* that both solvents can extract.

Table 15 Experimental parameters and extraction yields for the three types of extraction made when using W. *indica* material.

Process	Mass of plant (g)	Solvent flow rate (g/min)	Volume of solvent (mL)	Time (h)	T (^{o}C)	P (bar)	Mass of extract (g)	Yield (%)
$ScCO_2$/EtOH (5 wt.%)	38.85	19.63	-	2	40	300	0.785	2.02
$ScCO_2$/EtOH (5 wt.%)	38.00	19.58	-	2	40	300	0.723	1.90
$ScCO_2$/EtOH (10 wt.%)	38.00	25.29	-	2	40	300	1.188	3.13
Soxhlet/EtOH	5	-	80	4 (8 cycles)	78	-	0.77	15.4
Soxhlet/EtOH	5	-	80	4 (24 cycles)	78	-	1.12	22.4
Soxhlet/EtOH *R_1	5	-	80	4 (22 cycles)	78	-	1.20	23.9
Infusion	2	-	200	-	100	-	0.50	25.0
Infusion *R_1	2	-	200	-	100	-	0.41	20.5

*R_1 = W. *indica* left in extractor after the extration with $scCO_2$/EtOH;

Table 16 W. *indica* extraction conditions and yields reported in the literature.

Process	Parts and mass of plant (g)	Volume of solvent (mL)	Time (h)	Yield (%)	REF
CH_2Cl_2 extract	Aerial parts (5)	50	0.50	1.30	[80]
CH_2Cl_2 extract	Roots (5)	50	0.50	0.60	[80]
CH_2Cl_2 extract	Aerial parts (61)	600	24.00	9.80	[65]
CH_2Cl_2 extract	Aerial parts (325)	3250	24.00	0.09	[65]
CH_2Cl_2 extract	Aerial parts (2000)	8000	24.00	0.60	[59]
EtOH extract	Roots (200)	-	72.00	11.14	[63]
H_2O extract	Aerial parts (5)	150	1.00	23.80	[80]
H_2O extract	Roots (5)	150	1.00	16.20	[80]
H_2O extract	Leaves (200)	1000	24.00	21.50	[67]
H_2O extract	Leaves (1200)	20000	2.00	1.20	[69]
H_2O extract	Aerial parts (6)	60	24.00	5.80	[59]
H_2O extract	Aerial parts (5)	50	1.00	10.50	[59]
MeOH extract	Aerial parts (5)	50	0.50	10.50	[80]
MeOH extract	Roots (5)	50	0.50	15.30	[80]
MeOH extract	Whole plant (1200)	41	4.00	4.00	[58]
MeOH extract	Leaves (200)	2000	1.00	10.41	[69]
MeOH extract	Leaves (200)	2000	1.00	10.13	[69]
Soxhlet/MeOH	Aerial parts (500)	-	-	15.30	[81]

3.2 Chemical composition analysis

3.2.1 Total phenolic compounds and antioxidant activity of extracts

To quantify the phenolic compounds in the extracts obtained, the Folin-Ciocalteu colorimetric method was used. Phenolic compounds are one of the main substances responsible for the antioxidant capacity of plant extracts. This property was also analysed using the DPPH assay. These methods were used as described in chapters 2.3.2 and 2.3.3, respectively.

When applying these methods to the extracts of both Carqueja and W. *indica* obtained with $scCO_2$, with or without co-solvent, a great difficulty arose in chosing an appropriate solvent to dissolve the extracts, due to the polarity of the compounds extracted by this process. The two methods require that the extract be soluble in polar solvents. In the case of an extract obtained with a low polarity solvent, the concentration of the extract can be lowered, to make sure the solubility limit is not exceeded, but this will probably result in too low an amount of the compounds of interest. Or a solvent with lower polarity can be used to dissolve the extract, but then when the polarity of the medium is increased in further steps of the method, precipitation can occur. **Table 17**, shows the operating conditions used, as well as the amount of total phenolic compounds and antioxidant activity values obtained.

Table 17 Extraction conditions, total phenolic compounds (TPC) and antioxidant activity, expressed as half maximum effective concentration (EC_{50}), of Carqueja and *W. indica* extracts.

Plant	Process	T (°C)	P (bar)	Yield (%)	Solvent	TPC ($mg_{GAE}/g_{extract}$)	TPC (mg_{GAE}/g_{plant})	EC_{50} (µg/mL)	EC_{50} ($g_{extract}/g_{DPPH}$)
Carq	$ScCO_2$/EtOH (5 wt.%)	40	300	1.23	EtOH	57 ± 6	0.7 ± 0.1	nd.	nd.
Carq	$ScCO_2$/EtOH (9 wt.%)	40	300	2.70	EtOH	63.5	1.7	high	> 6
Carq	$ScCO_2$/EtOH (14 wt.%)	40	300	4.36	EtOH	52 ± 6	2.3 ± 0.2	high	> 6
W. *indica*	$ScCO_2$/EtOH (5 wt.%)	40	300	1.90	CH_2Cl_2	10 ± 2	0.21 ± 0.04	high	> 6
W. *indica*	$ScCO_2$/EtOH (10 wt.%)	40	300	3.13	MeOH (80%)	8 ± 1	0.25 ± 0.03	high	> 6
Carq	Soxhlet/EtOH	78	-	20.4 ± 1.1	EtOH	67 ± 1	13.6 ± 0.6	31.8	0.96
W. *indica*	Soxhlet/EtOH	78	-	15.4	EtOH	77 ± 4	11.9 ± 0.6	53.4	1.62
W. *indica*	Soxhlet/EtOH	78	-	22.4	EtOH	39 ± 2	8.7 ± 0.5	179.3	5.81
Carq	Infusion/H_2O	100	-	22.2 ± 2.2	H_2O	118 ±7	24.1 ± 1.5	11.7	0.38
W. *indica*	Infusion/H_2O	100	-	25.0	H_2O	91 ± 2	22.7 ± 0.4	8.4	0.26

Comparing the values of **Table 17**, it can be concluded that the extractions usign infusion/water gave the best results, expressed by the highest TPC and the lowest EC_{50} values. Although the Carqueja infusion gave a higher TPC than the W*. indica* infusion, the latter exhibited a lower EC_{50}, which must be due to the type of compounds present.

For both W. *indica* and Carqueja, the extractions made with $scCO_2$ or with $scCO_2$/ethanol yielded very low TPC values and EC_{50} values that were too high to be determined correctly. As mentioned earlier, the two methods use polar solvents (water and methanol), which leads to believe that they are not the best methods to apply to extracts that have nonpolar or low polarity compounds. There were no reports on the determination of the antioxidant activity of Carqueja extracts obtained when using extraction with $scCO_2$. In this work, higher TPC values were always obtained for Carqueja than for W. *indica*.

In the case of Soxhlet/ethanol extractions, higher TPC values also corresponded to lower EC_{50} values, results being slightly better for Carqueja than for W. *indica*.

The Carqueja assays, in relation to TPC values, are in agreement with results reported in the literature, in which aqueous extraction yielded a greater amount of phenolic compounds compared to an ethanol extraction [30]. **Table 18** gives data found in the literature for the P. *tridentatum* species, with different parts of the plant and different types of extractions. For aqueous flowers extracts, 155 $mg_{gallic\ acid\ equiv}$ / $g_{Carqueja}$ and 50 ± 1 µg / mL were obtained in the literature [26,34,35], which are higher values than those obtained in these work. Some studies assessed the influence of the harvesting time and the extraction time in the preparation of the plant aqueous extracts, and verified that there is no direct relationship between the polyphenolic content and the antioxidant activity of the different extracts obtained [29]. For example, Luís *et al.* determined TPC values of methanolic extracts from ten Portuguese species. One of them was P. *tridentatum* (L.) Willk. Using the Folin-Ciocalteu colorimetric method, these authors found that leaves and stems of Carqueja had a lower phenolic content (113.6 ± 1.5 $mg_{gallic\ acid\ equiv}$ / $g_{Carqueja}$) than their flowers (171.4 ± 0.7 $mg_{gallic\ acid\ equiv}$ / $g_{Carqueja}$) [37]. These types of extracts gave the highest DPPH scavenging activity and reducing power. This might be explained by their specific profile in phenolic compounds, mainly dihydroflavonol and isoflavone derivatives [36].

It was also possible to find in the literature the contents in alkaloids, tannins and flavonoids of Caqueja and W. *indica* extracts (**Table 19**) [63]. For the extracts of W. *indica*, only radical scavenging activity (% RSA) values were found. For hexane extracts, the RAS values were 92.8% and 54.5% for the leaves and stems, respectively. Likewise, the methanolic extracts presented RAS values 38.1% and 26.3%, for leaves and stems, respectively [82].

Table 18 Extraction conditions, total phenolic compounds (TPC) and antioxidant activity, expressed as half maximum effective concentration (EC_{50}), of Carqueja extracts.

Process	**Parts, local and mass of plant (g)**	**Volume of solvent (mL)**	**Time (h)**	**Yield (%)**	Total Phenols	Antioxidant activity	**REF**
					TPC (mg_{GAE}/ $g_{extract}$)	**EC_{50} (µg/mL)**	
EtOH extracts	(10)	100	12.00	60.2 ± 0.225	22.4	61.7 ± 0.38	[29]
H_2O extracts	Flowers (1)	250	0.08	-	-	50 ± 1	[34,35]
H_2O extracts	Flowers (50), Castro Daire	1000	2.17	-	155	-	[26]
MeOH extract	Flowers (1)	25	1.00	-	-	180 ± 0.01	[33,34, 36]
MeOH extracts (70%)	Whole (200), Vila Real	-	0.50	-	-	150	[32]
Soxhlet/MeOH	S&L (100), Serra Da Estrela	1000	-	26.1 ± 1.7	113.6 ± 1.5	69.7 ± 11.9	[37]
Soxhlet/MeOH	Flowers (100), Serra Da Estrela	1000	-	26.1 ± 1.7	171.4 ± 0.7	26.1 ± 1.3	[37]

Table 19 Contents in akaloids, flavonoids, tannins and total phenolic compounds of extracts from Carqueja and W. *indica*, reported in the literature.

Process	**Parts, local Mass of plant (g)**	Alkaloids	Flavonoids	Tannins	Total Phenolic compounds	Antioxidant activity	**REF**
		(mg_{PNG}/ $g_{extract}$)	**(mg_{R}/ $g_{extract}$)**	**(mg_{GAE}/ $g_{extract}$)**	**TPC (mg_{GAE}/ $g_{extract}$)**	**EC_{50} (µg/mL)**	
Soxhlet/ MeOH	Carq S&L (100), Serra Da Estrela	12	22	43.4	113.6 ± 1.5	69.7 ± 11.9	[37]
Soxhlet/ MeOH	Carq flowers (100), Serra Da Estrela	12	48	55.3	171.4 ± 0.7	26.1 ± 1.3	[37]
EtOH extract	W. *indica* roots (200)	-	41±3.7	250.98±22	262.46±17	-	[63]

3.2.2 TLC assays

The TLC assay plates were used with the purpose of giving a first impression of compounds that can be found in Carqueja extracts. The process described in chapter 2.3.1 was followed. **Table 20** shows the conditions used in the TLC assays.

Table 20 Conditions of operation and results of TLC assays.

Cod	Solution	Solvent	Eluent	Universal stains	R_F*
CAH	carqueja extract, *n*-hexane	*n*-hexane	ethyl acetate (40 ml) + *n*-hexane (40 ml)	$KMnO_4$	a)0 b)0,96
CACO	carqueja extract, ethanol	ethyl acetate + ethanol	ethyl acetate (40 ml) + *n*-hexane (40 ml)	$KMnO_4$	
CAE	carqueja extract, $scCO_2$	*n*-hexane + dichloromethane	ethyl acetate (40 ml) + *n*-hexane (40 ml)	$KMnO_4$	a)0
PAZ	olive oil	*n*-hexane	ethyl acetate (40 ml) + *n*-hexane (40 ml)	$KMnO_4$	a)0,74
PACID	linoleic acid	*n*-hexane	ethyl acetate (40 ml) + *n*-hexane (40 ml)	$KMnO_4$	a)0,96
PLED	(+)-ledene (10-2)	*n*-hexane	ethyl acetate (40 ml) + *n*-hexane (40 ml)	$KMnO_4$	a)0,93
PCAR	trans-caryophyllene (10-1)	*n*-hexane	ethyl acetate (40 ml) + *n*-hexane (40 ml)	$KMnO_4$	a)0,93
CAH	carqueja extract, *n*-hexane	*n*-hexane	ethyl acetate (40 ml) + *n*-hexane (40 ml)	Phosphomolybdic acid	a)0 b)0,55 c)0,76 d)0,86 e)0,96
CACO	carqueja extract, ethanol	ethyl acetate + ethanol	ethyl acetate (40 ml) + *n*-hexane (40 ml)	Phosphomolybdic acid	a)0 b)0,55 c)0,76 d)0,86 e)0,98
CAE	carqueja extract, $scCO_2$	*n*-hexane + dichloromethane	ethyl acetate (40 ml) + *n*-hexane (40 ml)	Phosphomolybdic acid	a)0 b)0,03 c)0,51 d)0,76 e)0,86 f)0,98
PAZ	olive oil	*n*-hexane	ethyl acetate (40 ml) + *n*-hexane (40 ml)	Phosphomolybdic acid	a)0 b)0,16 c)0,22 d)0,63 e)0,96
PACID	linoleic acid	*n*-hexane	ethyl acetate (40 ml) + *n*-hexane (40 ml)	Phosphomolybdic acid	a)0,51 b)0,76 c)0,86 d)0,91
PLED	(+)-ledene (10-2)	*n*-hexane	ethyl acetate (40 ml) + *n*-hexane (40 ml)	Phosphomolybdic acid	a)0 b)0,52 c)0,76 d)0,83 e)0,86
PCAR	trans-caryophyllene (10-1)	*n*-hexane	ethyl acetate (40 ml) + *n*-hexane (40 ml)	Phosphomolybdic acid	a)0,93
CAE	carqueja extract, $scCO_2$	*n*-hexane + dichloromethane	ethyl acetate (10 ml)	3,4-Dinitrophenylhydrazine	a)0 b)0,09 c)0,91
PPYR	Pyrogallol	ethyl acetate	ethyl acetate (10 mL)	3,4-Dinitrophenylhydrazine	a) 0,73
PGAL	Gallic acid	ethyl acetate	ethyl acetate (10 mL)	3,4-Dinitrophenylhydrazine	

PCAN	Borneol	ethyl acetate	ethyl acetate (10 mL)	3,4-Dinitropheny lhydrazine	
CAE	Carqueja extract, ethanol	ethyl acetate + ethanol	ethyl acetate (10 mL)	Phosphomol ybdic acid	a)0 b)0,65 c)0,91
PPYR	Pyrogallol	ethyl acetate	ethyl acetate (10 mL)	Phosphomol ybdic acid	a)0 b)0,22
PGAL	Gallic acid	ethyl acetate	ethyl acetate (10 mL)	Phosphomol ybdic acid	a)0,35
PCAN	Borneol	ethyl acetate	ethyl acetate (10 mL)	Phosphomol ybdic acid	a)0,70
CAE	Carqueja extract, $scCO_2$	*n*-hexane + dichloromethane	ethyl acetate (9,5 mL) + methanol (0,5 mL)	3,4-Dinitropheny lhydrazine	a)0 b)0,09 c)0,89
PPYR	Pyrogallol	ethyl acetate	ethyl acetate (9,5 mL) + methanol (0,5 mL)	3,4-Dinitropheny lhydrazine	a)0,77
PGAL	Gallic acid	ethyl acetate	ethyl acetate (9,5 mL) + methanol (0,5 mL)	3,4-Dinitropheny lhydrazine	
PCAN	Borneol	ethyl acetate	ethyl acetate (9,5 mL) + methanol (0,5 mL)	3,4-Dinitropheny lhydrazine	a)0,89
CAE	Carqueja extract, ethanol	ethyl acetate + ethanol	ethyl acetate (9,5 mL) + methanol (0,5 mL)	Phosphomol ybdic acid	a)0 b)0,13 c)0,95
PPYR	Pyrogallol	ethyl acetate	ethyl acetate (9,5 mL) + methanol (0,5 mL)	Phosphomol ybdic acid	a)0 b)0,80
PGAL	Gallic acid	ethyl acetate	ethyl acetate (9,5 mL) + methanol (0,5 mL)	Phosphomol ybdic acid	a)0,40
PCAN	Borneol	ethyl acetate	ethyl acetate (9,5 mL) + methanol (0,5 mL)	Phosphomol ybdic acid	a)0,85

*The Retardation factor is defined as the ratio of the distance migrated by the centre of a spot to the distance migrated by the solvent front. Each letter corresponds to a dash in the plate, in ascending order.

When exposing these TLC plates with the Carqueja extracts to UV light (254 nm), the silica gel fluoresces. It is noticeable, in all the extracts, a dark blue spot which only appears when we are in the presence of an organic molecule. These are also visible under visible light (366 nm), although less noticeably than in the latter case (Figure 32).

TLC assays identified in all the samples free fatty acids, triglycerides and terpenes. The Carqueja extract obtained with Soxhlet/ethanol had phenolic compounds (Figure 33). No traces of ketones were detected. Victor *et al.* reported a phytochemical screening of a Carqueja water extract that presented alkaloids, phenolic compounds (including flavonoids) and glycosilated terpenoids [28].

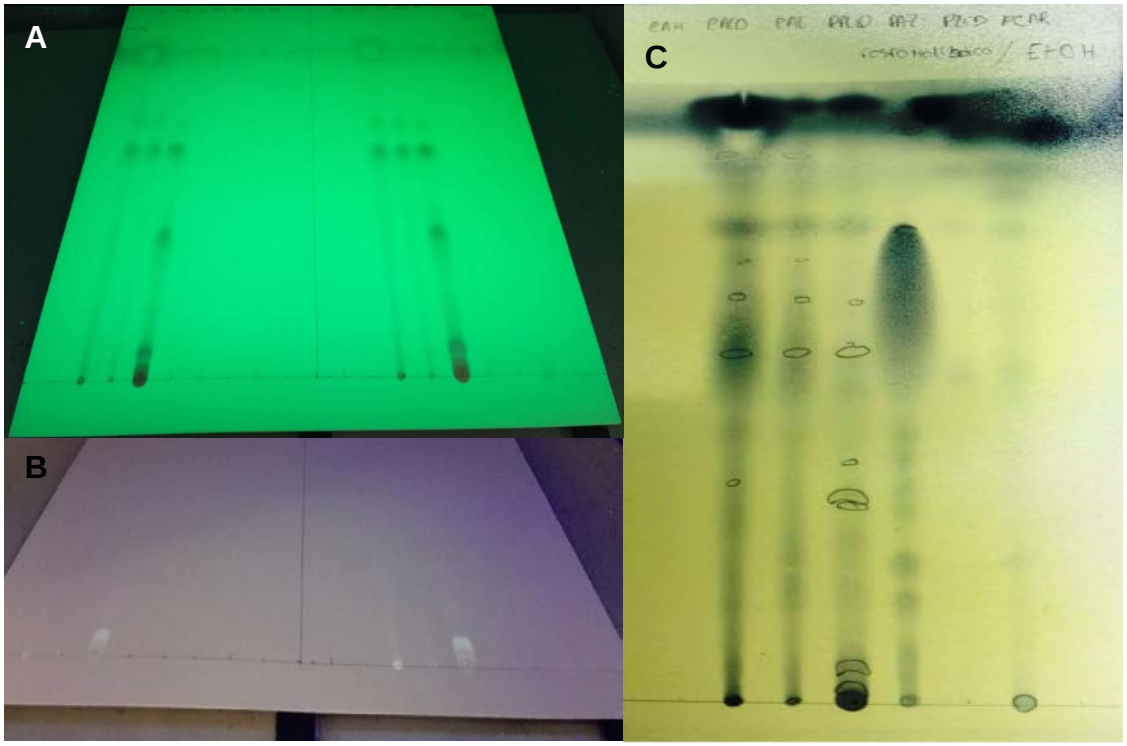


Figure 32 (A) Carqueja extracts observed under UV light (B) and visible light. (C) Carqueja extracts after spraying with the universal stain phosphomolybdic acid.

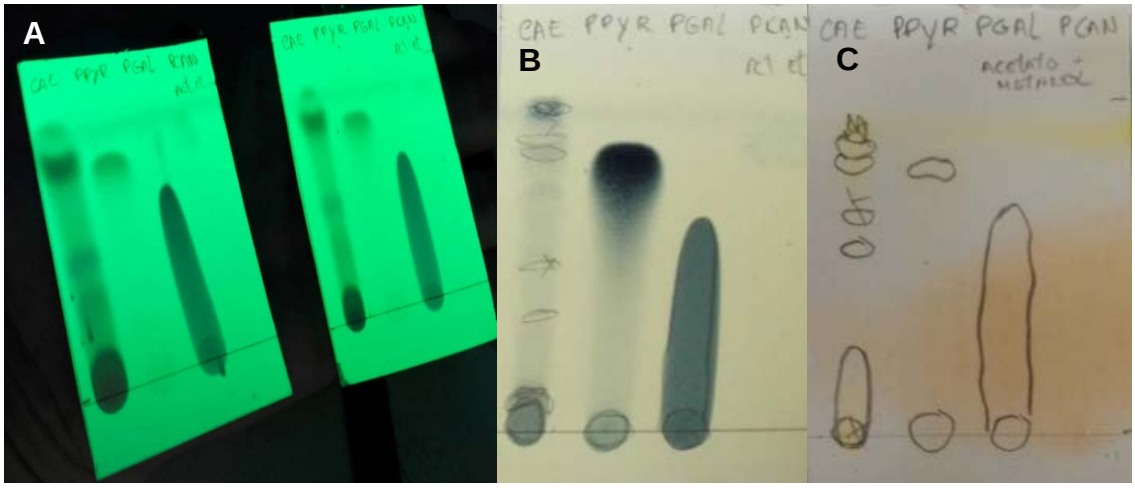


Figure 33 (A) Carqueja extract obtained with ethanol, observed under UV light. (B) Same Carqueja extract after spraying with the universal stains phosphomolybdic acid and (C) 3,4-dinitrophenylhydrazine.

3.2.3 Chromatography analysis. High performance liquid chromatography (HPLC) and gas chromatography – mass spectrometry (GC-MS).

The extracts obtained and described so far, from Carqueja and from W. *indica*, were sent to Merck (Darmstadt, Germany), for chromatographic analysis. The results obtained are summarized below.

- **Carqueja Extracts:**

In the $scCO_2$ extraction extract obtained at 500 bar, 6 peaks were detected at 230 nm, for the conditions presented in Appendix E. In the attempt to identify these compounds, by resorting to literature, the suggested structures are 5,7-Dihydroxychroman-4-one (RT UV: 13.790 min and Molar Mass: 180 g/mol), 3‘,4‘,5,7-tetrahydroxy-3-methylflavone or derivatives (RT UV: 20.310 min and Molar Mass: 300 g/mol) and 5,7-dihydroxy-2-(4- hydroxyphenyl)-3-methyl-chromen-4-one or Biochanin A or Prunetin - this depends where the methoxygroup is linked in the molecule (RT UV: 23.548 min and Molar Mass: 284 g/mol).

The chromatograms of extracts obtained by Soxhlet/*n*-hexane and by $scCO_2$ extraction (350 and 500 bar) look very similar when comparing the total number of peaks for the conditions presented in Appendix C. The area of these peaks is the highest in the $scCO_2$ extract at 500 bar.

The $scCO_2$/ethanol extract was analyzed, for different extraction times, and compounds were detected at 230 nm. As the extraction time increased, so did the number of peaks. Comparing the $scCO_2$ extraction with and without co-solvent, the one with co-solvent led to a higher number of peaks for the conditions presented in Appendix D.

By using infusion/water or Soxhlet/ethanol, a higher amount of peaks in number and area is obtained in extracts. At 255 nm, 21 peaks were detected in extracts obtained by infusion/water and 8 peaks for Soxhlet/ethanol extracts.

Additionally, Carqueja extracts were compared as regards the presence of a target compound, namely genistein. A 4-point-calibration (Appendix F) for genistein was built, for the conditions presented in Appendix D.

The results showed the presence of genistein in all the extracts analyzed, except those obtained by $scCO_2$ extraction, with or without co-solvent, as quantified in **Table 21**. The amounts of genistein in Carqueja ranged between 0.3-0.6 wt.%, and the extracts with the highest amount of genistein were those obtained by Soxhlet/ethanol.

Table 21 Content of genistein in Carqueja.

Sample name	Infusion	Soxhlet/EtOH	Soxhlet/EtOH after $scCO_2$ at 500 bar	Infusion after $scCO_2$ at 500 bar	Soxhlet/EtOH after $scCO_2$/EtOH (9wt.%)	Infusion after $scCO_2$/EtOH (9wt.%)
Mass concentration of extract (mg/mL)	1.44	2.64	1.25	1.08	1.10	1.15
Mass concentration of genistein (mg/mL)	0.02	0.05	0.03	0.02	0.03	0.02
Mass % of genistein in extract	1.39	1.89	2.40	1.85	2.73	1.74
Mass of extract (g)	0.35	4.833	1.54	0.37	1.39	0.40
Mass of plant material (g)	2.00	24.95	6.50	2.00	6.50	2.00
Mass % of genistein in plant material	0.24	0.37	0.57	0.34	0.58	0.35

- **W. *indica* Extracts:**

A GC-MS analysis of extracts obtained by $scCO_2$/ethanol was made using the conditions shown in Appendix G, where 20 peaks were detected. The identification of those compounds is shown in Appendices H and I. HPLC analysis at 315 nm revealed 16 peaks for extracts obtained by infusion, and 7 peaks for extracts obtained by Soxhlet/ethanol. W. *indica* extracts were compared as regards the presence of a target compound, namely tiliroside. A 4-point-calibration (Appendix J) for trans-tiliroside was built, for the conditions presented in Appendix K.

Tiliroside was not identified in the $scCO_2$/ethanol extracts. However, it was identified in extracts obtained by infusion or Soxhlet/ethanol. The results obtained are shown in **Table 22**. The amounts of tiliroside in W. *indica* were up to 0.25%, and the extracts with the highest amount of tiliroside were those obtained by Soxhlet/ethanol.

Table 22 Content of tiliroside in W. *indica.*

Sample name	Infusion	Soxhlet/EtOH	Soxhlet/EtOH after $scCO_2$/EtOH	Infusion after $scCO_2$/EtOH
Mass concentration of extract (mg/mL)	1.22	1.05	1.54	1.15
Mass concentration of trans-tiliroside (mg/mL)	0.01	0.02	0.02	0.002
Mass % of trans-tiliroside in extract	0.82	1.90	1.30	0.17
Mass of extract (g)	0.50	0.77	1.20	0.41
Mass of plant material (g)	2.00	5.00	6.50	2.00
Mass % of trans-tiliroside in plant material	0.20	0.30	0.24	0.04

CHAPTER 4: Conclusions and Future Work

The aim of this work was the valorization of plant materials, namely Carqueja and W. *indica*, both with countless reported applications, mainly in the health area. For this valorization different extraction methodologies were used, with special focus on supercritical fluid technology. The chosen SCF was CO_2 due to its physico-chemical properties, namely its low T_c and P_c, chemical inertness, non-toxicity, relatively low cost and the fact it is generally recognized as safe.

$ScCO_2$ was used pure and with different contents of co-solvent ethanol. For Carqueja, the best extraction yield was obtained at 40ºC and 300 bar - 4.4% - when using 14 wt.% of ethanol. Similarly for W. *indica*, the highest extraction yield - 3.1% - was obtained at the same temperature and pressure, but with 10 wt.% of ethanol.

For comparison, extractions were also performed by Soxhlet, using ethanol and *n*-hexane, and by infusion. Of all the extractions made, the highest yields, for both plants, were obtained in infusions, namely 22.5% for Carqueja and 25% for W. *indica*. The extraction yields obtained when using Soxhlet/ethanol were similar, namely 21.5% for Carqueja and 22.4% for W. *indica*. These results are within those presented in the literature. When using Soxhlet/*n*-hexane with Carqueja, an extraction yield of 1.7% was obtained, which was similar to yields obtained with $scCO_2$, pure or with co-solvent ethanol. This can be explained by the greater similarity between $scCO_2$ and $scCO_2$/ethanol mixtures and *n*-hexane, than between $scCO_2$ and $scCO_2$/ethanol mixtures and pure ethanol, or water, as regards polarity/solvent strength.

The extraction yields obtained in the Soxhlet/ethanol assays, using Carqueja left in the extractor after extraction with $scCO_2$ (500 bar), and in the Soxhlet/ethanol assays, using W.*indica* left in the extractor after extraction with $scCO_2$/ethanol, are a little higher than the ones obtained from Soxhlet/ethanol assays, with both plant materials as supplied commercially. This indicates that $scCO_2$ extracts compounds that are different from those extracted by ethanol alone, showing that $scCO_2$ extraction may be used as a pre-step to concentrate certain species in the material. The opposite is true when infusion is applied to Carqueja and W.*indica* previously extracted with $scCO_2$. This indicates that there are certain compounds that both water and $scCO_2$ are able to extract, in spite of being very different solvent media.

Considering that both Carqueja and W. *indica* present polyphenols as major compounds, the extracts were analysed with the purpose of quantifying total phenolic compounds (TPC) and evaluating their antioxidant activity. The results obtained allow to conclude that extracts generated by infusion had both the highest TPC and the lowest EC_{50} values. Although the Carqueja infusion gave a higher TPC than the W. *indica* infusion, the latter exhibited a lower

EC_{50}, which must be due to the type of compounds present. For both W. *indica* and Carqueja, the extractions made with $scCO_2$ or with $scCO_2$/ethanol yielded very low TPC values, and EC_{50} values that were too high to be determined correctly. However, both assays require solubilization in polar media (water or methanol), which are not the most adequate to solvate compounds that apolar or low polarity media such as *n*-hexane or $scCO_2$ are able to extract.

In order to perform a preliminary characterization of Carqueja extracts, a TLC assay was performed. It allowed to identify the presence of free fatty acids, triglycerides and terpenes in $scCO_2$, Soxhlet/*n*-hexane and Soxhlet/ethanol samples. The extracts obtained with Soxhlet/ethanol had phenolic compounds. All the Carqueja extracts were visible in UV light and visible light.

A qualitative HPLC analysis of Carqueja extracts obtained by $scCO_2$ extraction allowed to detect 6 peaks, some of which were identified. By using infusion or Soxhlet/ethanol extraction, a higher amount of peaks in number and area were detected in the extracts, namely 21 and 8 peaks, respectively. Likewise, 20 peaks were detected and many of them were identified in W. *indica* extracts obtained in $scCO_2$ assays, using GC/MS. Moreover, using HPLC, the infusion extracts of W. *indica* yielded 16 peaks, while Soxhlet/ethanol extracts yielded 7 peaks. More information on the results of this analysis are given in Appendices D, E, F, H, I and J.

Two target compounds were quantified in Soxhlet/ethanol and infusion/water extracts from Carqueja and W. *indica*, namely genistein and tiliroside, respectively. The content of genistein in Carqueja ranged between 0.3-0.6 wt.%, whereas the content of tiliroside in W. *indica* was of ca. 0.25 wt.%. In both cases, the extracts with the highest amount of either of the target compounds were those obtained by Soxhlet/ethanol.

To determine if $scCO_2$ extraction of the two plant materials is worth investing in, the characterization of the extracts obtained using this technique must be performed. It is true that the yields of extraction obtained were always low, but this may not be an obstacle if there is enough value in the extract. Also as mentioned earlier, additional experiments should be performed to better analyze trends in the results, and to optimize extraction conditions. This work was merely a first approach to the $scCO_2$ extraction of Carqueja and W. *indica*.

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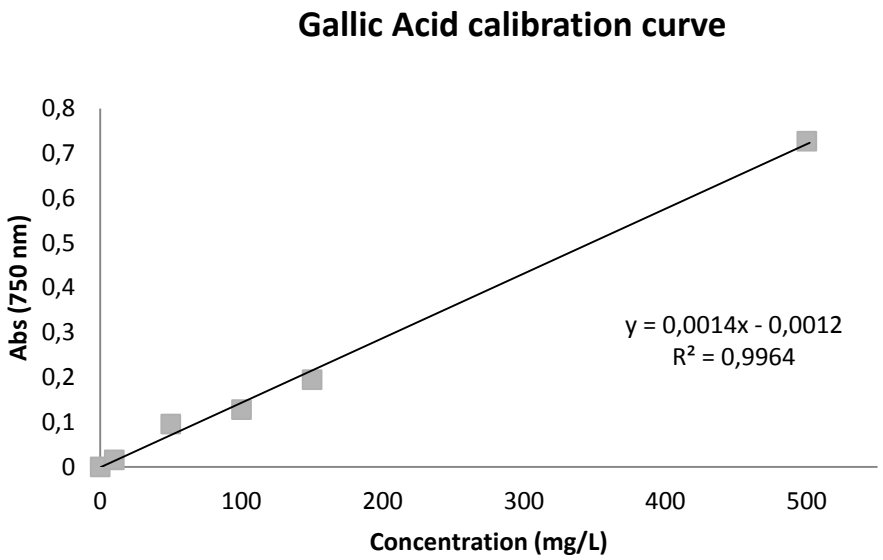
APPENDIX

Appendix A - Batches of packages of Carqueja flowers used in extractions:

- 01CAR465J171S
- 03CAR465J171S
- 05CAR465J171S

Appendix B - Calibration curve for the Folin-Ciocalteu method



Appendix C - HPLC Conditions used in the analysis of Carqueja extracts: $scCO_2$ at 350 and 500 bar, Soxhlet/EtOH and Soxhlet/Hexane, and Infusion. Information from Merck.

•Column: Chromolith Performance RP-18e 100mm, 4,6mm ID

•EluentA: Acetonitril+ 2% Water

•EluentB: Water+2% ACN+ phosphatebuffer

•Method: Tilirosid_Elite_15min_315nm_V2

Gradient:

Time (min)	EluentA (%)	EluentB (%)	Flow (mL/min)
0.0	5	95	2.5
6.0	5	95	5.0
10.0	10	90	5.0
28.0	63	37	5.0
30.0	80	20	5.0
32.0	80	20	5.0
36.0	5	95	5.0
37.5	5	95	5.0
38.0	5	95	1.0

Appendix D - HPLC Conditions used in the analysis of Carqueja extracts: $scCO_2$/EtOH and $scCO_2$ at 500 bar. Information from Merck.

•Column: Chromolith Performance RP-18e 100mm, 4,6mm ID

•EluentA: Acetonitril+ 2% Water

•EluentB: Water+2% ACN+ phosphate buffer

•Method: CornFlower_15min_Elite_M23_V2

Gradient:

Time (min)	EluentA (%)	EluentB (%)	Flow (mL/min)
0.0	5	95	2.5
6.0	5	95	5.0
10.0	10	90	5.0
28.0	63	37	5.0
30.0	80	20	5.0
32.0	80	20	5.0
36.0	5	95	5.0
37.5	5	95	5.0
38.0	5	95	1.0

Appendix E - HPLC Conditions used in the analysis of Carqueja extracts: $scCO_2$ at 500 bar. Information from Merck.

•Column: 259 – YMC-PACK ODS-A AA12S03-1546WTm 150 x 4.6, S-3µm, 12nm No.0415174769 (W)

•Solvent A: H2O/0.1% TFA (Id-Nr.: 2017.1212.MK01)

•Solvent B: ACN/0.1% TFA (Id-Nr.: 2018.0104.MK01)

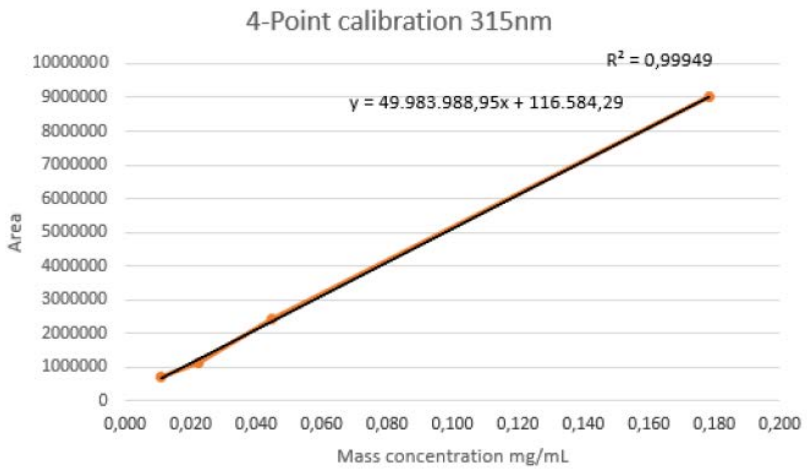
•Injection volume: 10 uL

•Sample Amount: 1.15 mg dissolved in 1 mL MeOH (Art.1.06035.2500, Ch.I0892835)

Gradient:

Time (min)	A (%)	B (%)	Flow (mL/min)
0.0	95	5	1.0
6.0	95	5	1.0
10.0	90	10	1.0
28.0	37	63	1.0
30.0	20	80	1.0
32.0	20	80	1.0
36.0	95	5	1.0
37.5	95	5	1.0
38.0	95	5	1.0

Appendix F – Calibration curve for genistein. Information from Merck.



Appendix G - GC-MS data for extracts of W. *indica* obtained by $scCO_2$ extraction. Information from Merck.

•Column: VF-5ms 30m x 0.25mm ID DF=0.25µm

•Column temperature: 50°C (1min) - 320°C (26.2min)

•Temperature program: 8 degr/min

•Constant Flow: 1.0 ml/min

•Injector temperature: 280°C

•Injection volume: 1µl Split Ratio 1:25

•Sample concentration:

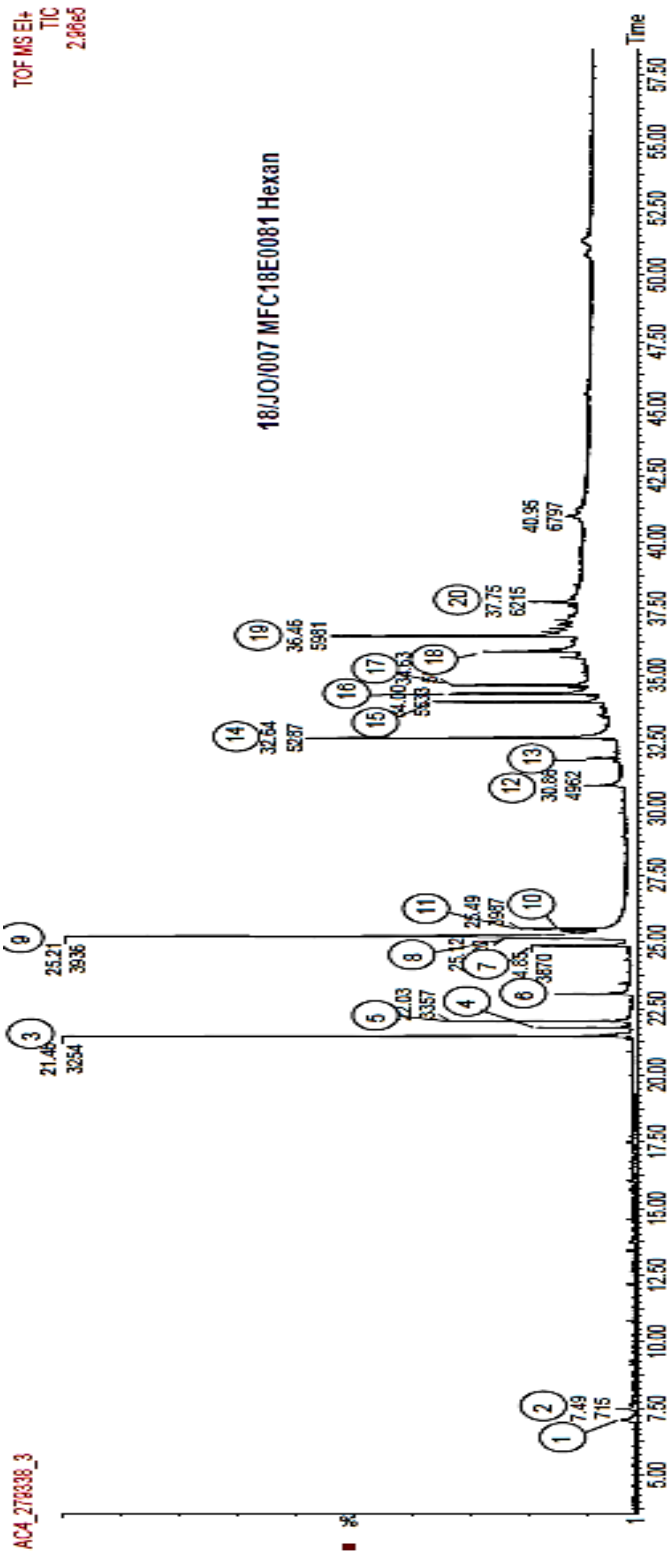
(AC4_279337_3) 5mg in 500µl hexane

(AC4_279338_3) 5mg in 500µl hexane

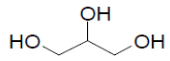
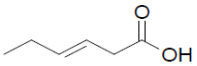
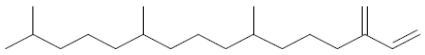
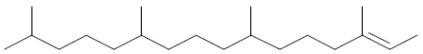
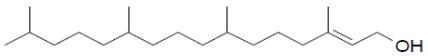
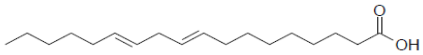
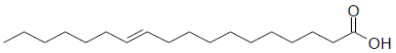
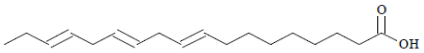
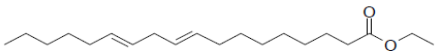
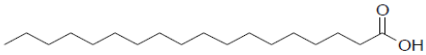
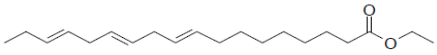
•Instrument: GCT-P CAB096 (HP6890 GC)

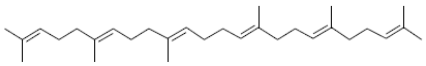
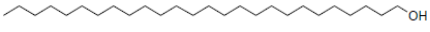
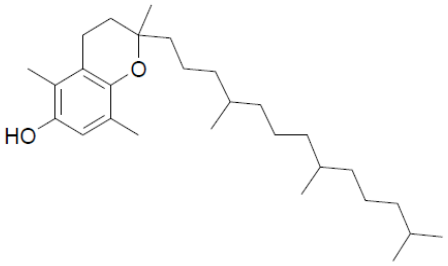
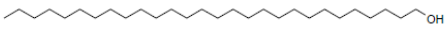
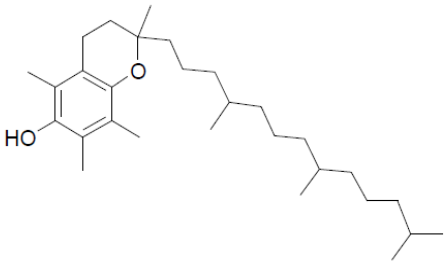
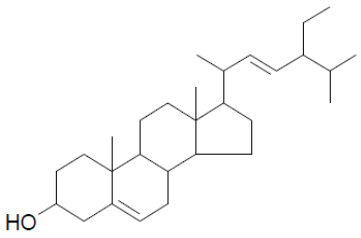
•MS File: EI800_FID_61min

Appendix H - GC-MS data for extracts obtained for W. *indica* by $scCO_2$ extraction. Information from Merck.

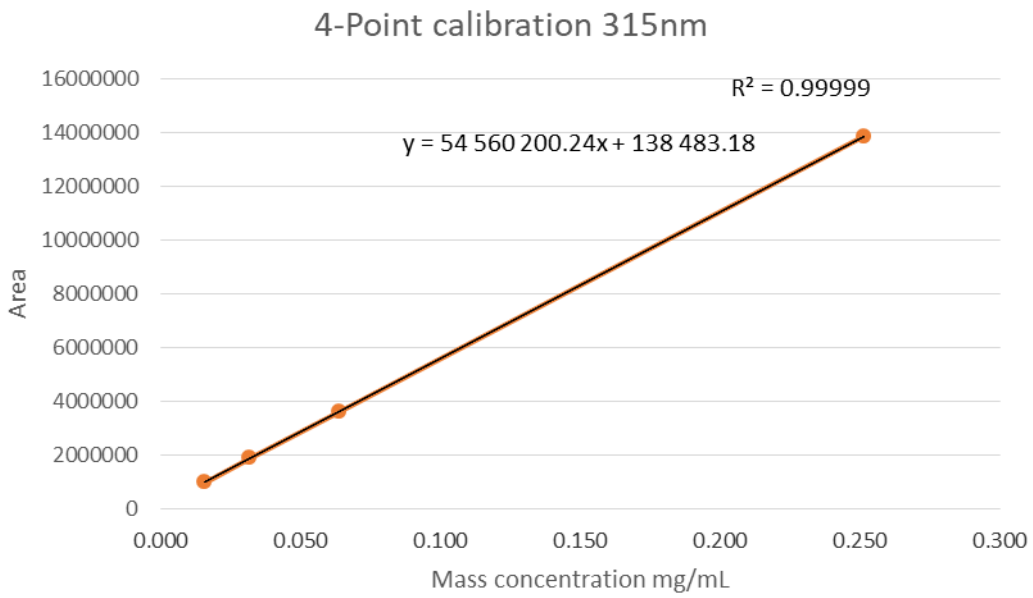


Appendix I - GC-MS data for W. *indica* extracts obtained by $scCO_2$ extraction. Information from Merck. Under mass spectrometric conditions of electron impact, the following compounds are detected:

Peak#	Scan# (TIC)	Ret. Time [min] (TIC)	Mass	Proposed structure	Comment
1.	635	7.05	92.047		$C_3H_8O_3$ or isomer
2.	715	7.49	114.068		$C_6H_{10}O_2$ or isomer
3.	3250	21.44	278.297		$C_{20}H_{38}$ or isomer
4.	3268	21.54	280.313		$C_{20}H_{40}$ or isomer
5.	3310	21.77		Isomer to Peak 3, M:278	
6.	3353	22.00		Isomer to Peak 3, M:278	
7.	3870	24.85	(296.308)		$C_{20}H_{40}O$ or isomer no molecular ion
8.	3921	25.13	280.240		$C_{18}H_{32}O_2$ or isomer position of the double bonds not certain
9.	3929	25.17	(282.256) AND 278.225	 coelution with: 	$C_{18}H_{34}O_2$ or isomer $C_{18}H_{30}O_2$ or isomer position of the double bonds not certain
10.	3974	25.42	308.272 AND 284.272	Coelution:  	$C_{20}H_{36}O_2$ or isomer $C_{18}H_{36}O_2$ or isomer position of the double bonds not certain
11.	3988	25.50	306.256		$C_{20}H_{34}O_2$ or isomer position of the double bonds not certain
12.	4962	30.86	(354.386)		$C_{24}H_{50}O$ or isomer no molecular ion

Peak#	Scan# (TIC)	Ret. Time [min] (TIC)	Mass	Proposed structure	Comment
13.	5150	31.89	410.391		$C_{30}H_{50}$ or isomer Squalene
14.	5283	32.62	(382.417)		$C_{26}H_{54}O$ or isomer no molecular ion
15.	5527	33.97	416.365		$C_{28}H_{48}O_2$ or isomer
16.	5589	34.31	(410.449)		$C_{28}H_{58}O$ or isomer no molecular ion
17.	5643	34.60	430.381		$C_{29}H_{50}O_2$ or isomer
18.	5876	35.88	412.371	Possible by MS: 	$C_{29}H_{48}O$ or isomer

Appendix J – Calibration curve for tiliroside. Information from Merck.



Appendix K – HPLC Conditions used in the analysis of W. *indica* extracts: Infusion and Soxhlet/ethanol. Information from Merck.

Time (min)	EluentA (%)	EluentB (%)	Flow (mL/min)
0.0	5	95	2.5
10.0	5	70	2.5
13.0	30	95	2.5
15.0	5	37	2.5