# Valorisation of *Cistus ladanifer* L. biomass as a source of compounds for bio-based industries

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Thesis presented to

the School of Technology and Management of Polytechnic Institute of Bragança for the Double Diploma Degree in Chemical Engineering

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Bragança

2021

This work will be funded under the project from the Bio Based Industries Joint Undertaking (JU) under grant agreement No 887917 BeonNAT. The JU receives support from the European Union's Horizon 2020 research and innovation programme and the Bio Based Industries Consortium

**ACKNOWLEDGEMENTS**

First of all, I would like to express my sincere gratitude to my supervisor Professor Joana Amaral for her consistent support and guidance during the running of this project. Your insightful feedback pushed me to sharpen my thinking and brought this work to a higher level. I am extremely grateful to Professor Sandrina Heleno whose expertise was invaluable in formulating the research questions and methodology, without your help and wise guidance this project would have not been the same. Further, I would like to thank Professor Lillian Barros who inspired all the development of this project. I would like to say a special thank you to Professor Khalil Zaghdoudi whose guidance, support and encouragement has been invaluable throughout this study. I also wish to thank Virginie Xiavier who have been a great source of support understanding and help during the laboratory work. To conclude, I cannot forget to thank my family and friends for all the unconditional support in this very intense academic year.

**To all, my thanks!**

# Abstract

*Cistus ladanifer* L., a perennial shrub from the Cistaceae family that can be found in abundance in the Mediterranean's marginal fields, is known to produce a valuable compound-rich resin called labdanum. *C. ladanifer* has also been extensively used for different purposes such as cosmetics, traditional medicine, feed, phytoremediation, and biofuel production.

The present work aimed at studiyng the chemical composition of different products obtained from the plant, namely the essential oil of and different concretes prepared from the leaves and buds using two different solvents, hexane and ethanol. Three different extraction methods were used for obtaining the concretes, namely soxhlet, microwave assisted extraction and ultrasound assisted extraction. The chemical composition was analysed using gas chromatography coupled to mass spectrometry (GC–MS). Moreover, the obtained ethanol and hexane concretes were assayed for their antioxidant, antimicrobial and antifungal activities. Most of the compounds identified in the essential oils from the buds and leaves belong to different groups of terpene and terpenoid compounds. In the concretes, besides terpene compounds different hydrocarbons were detected in high amounts. Nevertheless, both in the hexane and ethanol concretes the majority of the peaks were not identified. In general, the highest extraction yield was obtained by soxhlet (26.1± 0.8 % and 29±1 % for leaves and buds respectively). However microwave extraction method was privileged for the optimization studies since it requires less time and energy compared to Soxhlet and allowed obtaining extracts with higher content of viridiflorol.

The antioxidant potential was determined by applying two different methods namely the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the reduction power assays. The ethanolic concrete obtained from the leaves using microwave gave the best results in DPPH assay ($EC_{50}$=0.152 ± 0.005 mg/mL) while for reducing power better results were obtained for the ethanolic extract from the buds using ultrasound assisted extraction ($EC_{50}$=0.15 ± 0.01 mg/mL).

All the tested extracts revealed inhibitory activity against the tested Gram-positive and Gram-negative bacteria, with the exception of *Pseudomonas aeruginosa*.



**Keywords:** Mediterranean rockrose, *Cistus ladanifer* L., essential oils, hydrolates, extraction, phenolic compounds, antibacterial activity, antioxidant activity

# Resumo

*Cistus ladanifer L*., um arbusto perene da família Cistaceae que pode ser encontrado em abundância nos campos marginais do Mediterrâneo, é conhecido por produzir uma valiosa resina rica em compostos denominada lábdano. *C. ladanifer* também tem sido amplamente utilizada para diferentes fins, como na indústria cosmética, medicina tradicional, em rações, fitorremediação e produção de biocombustíveis.

O presente trabalho teve como objetivo estudar a composição química de diferentes produtos obtidos da planta, nomeadamente o óleo essencial e diferentes concretos preparados a partir das folhas e botões florais utilizando dois solventes distintos, hexano e etanol. Três diferentes métodos de extração foram usados para a obtenção dos concretos, nomeadamente soxhlet, extração assistida por micro-ondas e extração assistida por ultrassom. A composição química foi analisada por cromatografia gasosa acoplada a espectrometria de massa (GC-MS). Além disso, os concretos de etanol e hexano foram avaliados quanto às suas atividades antioxidante, antimicrobiana e antifúngica. A maioria dos compostos identificados nos óleos essenciais dos botões florais e folhas pertencem a diferentes grupos de terpenos e compostos terpenóides. Nos concretos, além dos compostos terpénicos, diferentes hidrocarbonetos foram detectados em grandes quantidades. No entanto, em ambos os concretos a maioria dos picos cromatográficos não foi identificada. Em geral, o maior rendimento de extração foi obtido por soxhlet (26,1 ± 0,8% e 29 ± 1% para folhas e botões, respectivamente). Porém, o método de extração por microondas foi privilegiado para os estudos de otimização, pois requer menos tempo e energia em relação ao Soxhlet e permitiu a obtenção de extratos com maior teor de viridiflorol.

O potencial antioxidante foi determinado pela aplicação de dois métodos diferentes, nomeadamente a captação do radical 2,2-difenil-1-picrilhidrazil (DPPH) e o poder redutor. O concreto etanólico obtido das folhas em micro-ondas apresentou os melhores resultados no ensaio DPPH (EC50=0,152 ± 0,005 mg/mL), enquanto para o poder redutor o melhor resultado foi obtido para o extrato etanólico dos botões florais utilizando extração assistida por ultrassom (EC50=0,15 ± 0,01 mg/mL).

Todos os extratos testados revelaram atividade inibitória contra as bactérias Gram-positivas e Gram-negativas testadas, com exceção de Pseudomonas aeruginosa.



**Palavras-chave:** Esteva do Mediterrâneo, *Cistus ladanifer L*., óleos essenciais, hidrolatos, extração, compostos fenólicos, atividade antibacteriana, atividade antioxidante.

**Table of contents**

**List of figures**

**List of tables**

# I. Essential oils and hydrolates

## I. 1. Essential oils

Essential oils are oily liquids normally obtained from raw plant materials (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots) through distillation with water and/or steam. Essential oils chemical composition can include a variety of compounds of low molecular weight such as phenolics, aldehydes, ethers, and ketones (Wani et *al*., 2020). The main chemical group present in essential oils are terpenes, which are secondary metabolites of plants that can be classified according to the number of constituting isoprene units, each containing 5 carbons. Monoterpenes (C10) and sesquiterpenes (C15), containing 2 and 3 units respectively, are the most frequent classes. Usually, a number as high as 100 to 250 components can be identified in essential oils, although only a few are present in high amounts. Major compounds are generally 2 to 5 components that together make up about 50% to 60% of the oil. However, in some cases there is only one main compound, which can correspond to more than 50% of the oil (De Groot & Schmidt, 2016). For example, compounds such as β-caryophyllene and limonene have been described in amounts of 98% and 97%, respectively. In addition, some compounds are frequently found in different essential oils, with chemicals like limonene, α-pinene and linalool being described as essential constituents in more than 20 different oils (De Groot & Schmidt, 2016).

The composition of essential oils can be affected by many factors such as species, cultivation methods, geographic and climate conditions. For this reason, different compounds and different amounts of the same compounds can be found in plants from the same species (Russo et *al*., 2012).

The average yield of essential oil distilled from aromatic plants is usually lower than 5% (w/w) and as a consequence, substantial solid residues are produced as a result of essential oil extraction processes. These residues are particularly rich in polyphenols, which can be used in food and feed as health-promoting agents or as anti-aging ingredients in cosmetic products to improve the overall viability of aromatic plants (Sánchez-Vioque et al., 2013).

## I. 2. Hydrolates

Hydrolates are complex blends that contain traces of essential oils. They are cheap, easy to produce and less toxic than their respective essential oils (Prusinowska et *al*., 2016). During the process of essential oils distillation, hydrolates (also known as hydrosols, floral waters and aromatic waters) persist dissolved in the distillation water, generally in very low amounts

(Marino et *al*., 2020). In the study conducted by (Marino et al., 2020)), the hydrolate's essential oil content of *Coridothymus capitatus* (L.) was measured gravimetrically and was found to be 0.1403% (v/v). In this case, carvacrol (93.11%) and thymol (6.34 %) were the key hydrolate identified components. Camphor (0.04%) and terpineols (such as α-terpineol (0.08%), terpinen-4-ol (0.15%) and endo-borneol (0.18%)) were also present.

In another study performed by Tavares et *al*. (2020), terpinen-4-ol (21-31 %), *p*-cymene-8-ol (11-16 %), umbellulone (48 %), camphor (1-11 %) and cis-3-hexen-1-ol (0.1-13 %), were the main volatiles identified in the hydrolates of *Cupressus lusitanica* Mill..

Due to the presence in lower amounts of compounds that are biologically active, hydrolates have more delicate aromas than the corresponding essential oils. Hydrolates are used in the food industry and in the manufacture of soaps, creams, lotions and air fresheners (El et *al*., 2014), being considered very important components of cosmetic products because of their fascinating aromatic characteristics. They are well tolerated by the skin due to their low concentration in terpene and sesquiterpene hydrocarbons, thereby avoiding irritation (Śmigielski et *al*., 2013). Hydrolates are commonly used in lotions, body tonics, after-shaves, face creams and as components of medicinal and rejuvenating facials. In addition, they appear to stimulate the human mind in massage, inhalation and aromatherapy (Śmigielski et *al*., 2013).

## I. 3. Extraction methods

There is a variety of extraction methods that can be used to extract essential oils. In fact, the most popular and conventional methods are hydrodistillation and steam distillation. Nevertheless, new methods such as microwave assisted extraction (MAE), ultrasound assisted extraction (UAE) and supercritical $CO_2$ extraction (SFE) have been pursued in order to decrease the extraction time, reduce the costs, improve the quality of the extracts and increase the extraction yields (Gavahian et *al*., 2015).

### I.3.1. Hydro and steam distillations

Hydrodistillation (water distillation), is considered the oldest method of distillation. This method protects the oils because it doesn't damage fresh and dry plant parts by heating (Sovová & Aleksovski, 2006). In fact, the surrounding water acts as a barrier to prevent them from overheating (Sefidkon et al., 2007). The distillation is carried out with the use of boiling water where the botanical material is completely immersed (contrary to the steam distillation). The essential oil is then transported via vapour and accumulates in the water due to the difference

in density, as it hits the Florentine container which is the collecting vessel (Sovová & Aleksovski, 2006). At a laboratory scale, hydrodistillation is generally performed using a Clevenger system as depicted in Figure 1. However, this process has some drawbacks, such as long-time extraction, losses of volatile compounds, and low extraction efficiencies (Gavahian et *al*., 2012). The Clevenger method of hydrodistillation is advised by the European Pharmacopoeia's third edition for assessing essential oil yields. It has a cohobage mechanism that allows the condensates to be recycled. This method is perfect for extracting petals and flowers (such as rose petals) because it prevents plant material from compacting and clumping while extraction is being performed (Asbahani et *al*., 2015).

The hydrodistillation process is affected by some parameters such as the power, solid/liquid ratio and extraction time. In fact, if the temperature is high, the rate of extraction is fast contrary to the use of low temperatures. On the other hand, solid/liquid ratio, which is the quantity of sample (g) per volume of solvent (in this case water, mL), is an important parameter in hydrodistillation. According to the available literature, the best ratio of sample to solvent is normally 1:10 according to the European Pharmacopeia. Another important parameter is the time that should be enough to extract all the essential oil existing in the plant sample (Samadi et *al*., 2017).

Hydrodistillation was used in the work of Samadi et al., (2017) to extract essential oil from *Aquilaria malaccensis* Lam. leaves in order to identify its chemical composition.

Another important technique, which is the most common method used by the industry for extracting essential oils from plant matter, is the steam distillation. In fact, it is a technique for separating high boiling mixtures or separating a substance from a non-volatile impurity. It's used to separate mixtures at a lower temperature than their constituents' boiling points. The biggest benefit of using steam distillation is that the gases are quickly condensed in water. Furthermore, steam also displaces atmospheric oxygen, shielding the material from oxidation (Hanci et *al*., 2003). In this technique, high-pressure steam is pumped into a gap under a perforated grid, which supports the sample of botanical material. The steam heats and saturates the plant as it moves through it. The resulting vapour, which is a combination of steam and essential oil vapours, escapes through the top and is led to a condenser (Sovová & Aleksovski, 2006). For instance, the steam distillation is commonly used to extract hydrolates from *Lavandula angustifolia* Mill. (Prusinowska et al., 2016).

### I.3.2. Microwave assisted extraction (MAE)

Microwave-assisted hydrodistillation (MAE) is a sophisticated hydrodistillation method using a microwave oven for the extraction of bioactive compounds. Compared to common hydrodistillation, this extraction technique requires less energy, less time of extraction, reduces the operation costs and improves the extraction yield, without affecting the composition of the essential oils with its irradiations (Golmakani & Rezaei, 2008). It is a method of extracting soluble products from a wide variety of materials using microwave energy into a fluid (Sadeghi et *al*., 2017). In medicinal plant research the interest of this technique has increased significantly over the past 5-10 years, owing to its inherent benefits as a specific heating mechanism, modest capital costs and its good performance using atmospheric conditions (Sadeghi et *al*., 2017).

Microwaves are a form of non–ionizing electromagnetic energy generated by two perpendicular oscillating fields: the electric and the magnetic field. They can be used as carriers of information or as vectors of electricity. This energy is ranging at frequencies from 300 MHz to 300 GHz, an particularly in commercial devices, MAE with the frequency 2450 MHz can be used and distributed in the form of waves capable of penetrating biomaterials (Waseem & Low, 2015).

In general, microwave assisted extraction mechanism should include three sequential phases, which are: i) the separation of solutes from active sample matrix sites at high temperature, ii) the propagation of solvents through the sample matrix, and iii) the migration of the target compounds from the sample matrix to the solvent (Sadeghi et al., 2017).

MAE is used to extract essential oils which are thermo-sensitive complexes, as an example, the extraction of Puerarin from the *Radix puerariae* (Willd.) Sanjappa & Pradeep herb has been accomplished in just 1 min. Also, *Cinamomum iners* Reinw essential oils, were obtained by MAE, where linalool was found as the major isolated portion with high yields of oil (Waseem & Low, 2015).

MAE invention has contributed to the development of a large range of variants, including microwave assisted hydrodistillation, solvent free microwave extraction, microwave hydrodiffusion and gravity and compressed air microwave distillation (Asbahani et *al*., 2015). Also, closed vessel system (under regulated temperature and pressure) or an open vessel system (at atmospheric pressure) may be used to apply microwave energy to the samples (Waseem & Low, 2015).

- **Microwave assisted hydrodistillation**

Based entirely on the conventional hydrodistillation principle, a portion of the hydrodistillation assembly line is put in a microwave oven. The matrix is inserted in a reactor with water that has been put inside the microwave oven. Outside the oven is the refrigeration system and the portion that is intended to recover essences. This method has already been used to extract essential oils from a range of aromatic plants and spices, for example *Thymus vulgaris* L. and *Zataria multiflora* Boiss, for which the essential oils were extracted with a yield of 2.5% and 3.7% respectively (Chemat et *al*., 2013).

- **Solvent free microwave extraction**

This technique is based on combining dry distillation with microwave heating energy (Asbahani et *al*., 2015). The fresh matrix is inserted in a microwave reactor without the addition of any solvent in this process. The plant's glands containing essential oil are broken when the raw material is heated with water. This step allows the essential oil to be released, which is then pushed forward by steam produced from matrix water. A cooling system outside the microwave oven collects the distillate, which is made up of water and essential oil, to constantly condense (Chemat et *al*., 2013). This process takes just 30 minutes to separate and concentrate volatile compounds, whereas traditional hydrodistillation usually takes two hours (Asbahani et *al*., 2015).

Through this technique, for instance, *Ocimum basilicum* L. and *Mentha crispa* L. essential oils were extracted with a yield of 0.029% and 0.095%, respectively (Chemat et *al*., 2013).

- **Microwave hydrodiffusion and gravity**

This method was developed to extract essential oils from a variety of matrices using microwave radiation and hydrodiffusion at atmospheric pressure (Chemat et *al*., 2013). Internal heating of water plant material dilates plant cells and allows glands and oleiferous receptacles to burst (due to a heating microwave action), allowing essential oils and plant water to escape the plant material. Extracts are guided from the top to the bottom of the microwave reactor to the cooling device by gravity (Asbahani et *al*., 2015). This approach has been used on a number of plants, including aromatic plants and citrus fruits such as *Menthe pulegium* L. and *Citrus limon* L. essential oils obtaining the yields of 0.95% and 1.6%, respectively (Chemat et *al*., 2013).

- **Compressed air microwave distillation**

This technique is known as the first microwave method for extracting essential oils from aromatic plants. This process is based on the steam entrainment principle, but instead of steam, compressed air is used to extract the essential oil. In the reactor, compressed air is continually pumped into the matrix, which is submerged in water and heated by microwaves. The steam is guided to a recovery vessel submerged in a refrigeration device situated outside the microwave oven after being saturated in volatile molecules. Water and aromatic molecules are condensed and recovered in identical amounts to a traditional method after just a few minutes (Chemat et *al*., 2013). This technique was used to extract essential oil from *Lippia sidoides* Cham leaves and was compared with steam distillation. Results showed that both oils tend to have the same overall composition (Craveiro et al., 1989).

### I.3.3. Ultrasound assisted extraction (UAE)

Ultrasound assisted extraction remains as a sustainable solution that calls for modest solvent and energy expenditure. In addition, since this technology enables its production under atmospheric pressure conditions and at room temperatures it is simple to manage, safe and reproductible (Soria & Villamiel, 2010; Vieira et *al*., 2013).

This technology can be used to obtain various phytochemicals from which phenolic compounds stand out (Medina-Torres et al., 2017). It is based on the acoustic cavitation theory, which can damage the cell walls of the plant matrix and thus facilitate the release of bioactive molecules (B. K. Tiwari, 2015). This extraction method involves the passage over the liquid media of a higher amplitude ultrasonic wave, which creates and breaks down the bubbles in a short time because of the high pressure and temperature produced within a very short time in the solvent. A powerful shock wave and high speed microjets produce the asymmetric collapse of the cavities. For instance, on the surface of a leaf, it generates cracks, divides, and ruptures the cell tissue. This will promote a higher contact of the solvent with the target compounds, allowing a more efficient recovery of the bioactives present in the matrix (Jadhav et al., 2020).

Three variables were evaluated in the extraction of essential oil from clove to optimize UAE parameters: extraction time, temperature, and plant concentration. In fact, the highest clove extract yield was obtained applying 45 minutes of extraction time, a temperature of 60°C and 5% plant concentration. These authors concluded that the most important factor affecting UAE yields was the temperature (Tekin et al., 2015). Comparing the extraction of the essential oil from *Melaleuca bracteata* F.Muell. leaves using traditional hydrodistillation extraction process, with the UAE under optimized extraction conditions, it was possible to state that this last

method presented a higher essential oil yield (4.55%), while the traditional hydrodistillation extraction process obtained an yield of 1.02% (Li et *al.*, 2018).

In another study, the UAE was used for the extraction of essential oils from apricot, almond and rice bran and the yields of extraction were 77, 87, and 88 % , respectively, using 4, 2, and 6 minutes of ultrasonication (Waseem & Low, 2015).

According to Dalla Nora & Borges (2017), when compared to traditional hydrodistillation, the use of ultrasonic pre-treatment allows the reduction of 70% of the extraction time. The use of extraction conditions such as low frequencies (20 to 50 kWz), time (20 to 40 min), allowed an increase in bioactive compound´s extraction, which improved the antioxidant and antimicrobial properties of essential oils.

### I.3.4. Supercritical $CO_2$ extraction

A fluid that has been heated and compressed beyond its critical pressure and temperature is considered as a supercritical fluid. Among the supercritical fluids, the emphasis is especially given on the use of supercritical carbon dioxide because it is cheap, non-toxic, and has a moderate critical pressure and low critical temperature (Erkey, 2000). Thus, supercritical carbon dioxide extraction is currently a widely used process for the extraction of different components. For example, $CO_2$ has been described as the most commonly used supercritical solvent to extract pharmaceutical compounds from different sources such as microalgae and eukaryotic photosynthetic microorganisms, that might be used to manufacture high-value compounds (such as hydrocarbons, astaxanthin, canthaxanthin, β-carotene) since those compounds can be obtained without thermal degradation and without toxic organic solvent contamination (Mendes et *al*., 2003).

Regarding the operating conditions, it is described that at 1071 psi and 31.1°C, carbon dioxide enters a supercritical state. If a molecule is in a supercritical state, it has both liquid and gas properties which is very useful because, like a gas, $CO_2$ can reach into small spaces, but can also dissolve and behave like a liquid solvent. Thus, the physical properties of supercritical $CO_2$ can be controlled, enabling it to achieve any target molecule.

The use of supercritical extraction (SFE) has been applied previously to several different matrices, with promising results. According to (Khajeh et al., 2004), the extraction yield of the essential oil of *Carum copticum* L. ranged from 1.0 to 5.8 % (w/w) when subjected to SFE, when compared to the hydrodistillation-based extraction yield that resulted on 2.8 % yield (v/w). This method is also interesting to extract flavouring compounds from vegetable

materials. For example, Iranian *Foeniculum vulgare* Mill. was obtained using SFE method, being possible to identify nine components that accounted for more than 99 percent of the obtained essential oil (Yamini et *al*., 2002).

Currently, SFE can be considered an industrial alternative to the processes that use solvent extraction and steam distillation, since it enables solvent strength and selectivity to be continuously changed by changing the density of the solvent (Khajeh et *al*., 2004).

## I. 4. Characterization of bioactive compounds

### I. 4.1. Gas Chromatography-Mass Spectrometry (GC-MS)

Gas Chromatography coupled to Mass Spectrometry (GC-MS) detection is a qualitative and quantitative analytical technique that combines the features of chromatography and mass spectrometry to identify different substances. Using this technique only volatile compounds or compounds that can be made volatile after derivatization can be analyzed.

For capillary GC-MS, the capillary column is inserted into the ion source by using a transfer line. As the GC effluent (individual compounds) elute, they enter the MS where a stream of electrons causes the fragmentation of ions that are bombarded. The mass of the fragment divided by the charge is the mass charge ratio (*m*/*z*) that reflects the fragment's molecular weight. Each fragment is then centred by a group of four electromagnets through a slit into the detector. The quadrupoles can be programmed to guide only selected fragments. Finally, the compounds can be identified based on the obtained mass spectrum, which works as a characteristic fingerprint (Sneddon *et al.*, 2007).

### I. 4.2. Liquid Chromatography-Mass Spectrometry (LC-MS)

Liquid Chromatography-Mass Spectrometry (LC-MS) is one of the most common methods used for the identification of compounds in complex chemical, biological and environmental systems (Yan et *a*l., 2020). Combining high-performance liquid chromatography (HPLC) and mass spectrometry (MS) gives one of the most efficient analytical methods of recent times to the analytical chemist.

Three basic steps are involved in LC-MS i) the chromatographic separation of compounds, ii) ionization, iii) ions separation and quantitation by MS. The key advantages of LC-MS are its precision and sensitivity as an analytical instrument. (Crutchfield et *al*., 2010).

LC-MS is the preferred technique for the separation and identification of semipolar secondary metabolites in plants. For example, LC-MS has recently been applied to detect secondary metabolites in root and leaves of *Arabidopsis* sp., to analyze metabolic alterations in

a light-hypersensitive tomato mutant and to evaluate potato tubers of various genetic origins and stages of development (Moco et *al.*, 2006).

## II. *Cistus ladanifer* L.

Cistaceae is a native Mediterranean family of nearly 200 shrub species, some of these being autochthonous species. Most members of this family are very perfumed and sweet-smelling, being highly valued for ornamental purposes and for the perfume industry. One of such species is *C. ladanifer*, which is a sticky shrub (Barrajón-Catalán et *al.*, 2010) of one to two meters tall, that blooms between April and June, and present leaves that secrete essential oils and resin. The resin, designated as labdanum, is primarily composed of flavonoids and terpenes (Benayad et *al.*, 2013). The most abundant flavonoids in the labdanum have been reported to be apigenin-4'-(O)methyl, kaempferol-3-(O)methyl and kaempferol-3,7-di(O)methyl. Other flavonoids, such as apigenin, apigenin-7,4'-di(O)methyl, apigenin-7-(O)methyl, kaempferol-3,7,4'-tri(O)methyl and kaempferol-3,4'-di(O)methyl) are minor constituents in the labdanum, contributing less than 10% to total flavonoids (Chaves et *al.*, 1993). Other studies describe the major compounds identified in labdanum as being terpenes, such as ledol, ledene and labdane skeleton derivatives (Greche et *al.*, 2009).

As will be mentioned, this plant species as many interest to different industries, yet its big downside is the possibility of fire promotion and propagation (Chase *et al.*, 2016).

### II. 1. Morphology and distribution

*C. ladanifer*, commonly known as rockrose, is a plant with white petals, linear-lanceolate to lanceolate leaves, (8)10(11) locules in the capsules, accentuated nervures in the upper surface, chromosome number equal to 2n=18, as with all the species of *Cistus* (Carlier *et al.*, 2008).

The rockrose is one of the Mediterranean basin's most common natural shrubs, widely extended in many countries such as Portugal, Spain, Greece, Italy, Algeria and Morocco, being generally present on poor soils and dry habitats. It is calculated that it spreads in the south/southwest of the Iberian Peninsula over an area of 2 million hectares. In Portugal, in particular, it extends throughout the country, especially in the central and southern regions (Alves-Ferreira, Duarte, Lourenço, et al., 2019).

## II. 2. Major bioactive phytochemicals

Phytochemicals have a significant role in the development and growth of plants. In fact, due to their presence, plants are protected from infectious agents such as insects and microbes and from traumatic events such as ultraviolet (UV) irradiation and high temperatures (Martinez et *al.*, 2017). They can be found in many parts of plants like stems, flowers, leaves, seeds, roots and grains (Liu, 2013).

Alkaloids, phenolic compounds, terpenoids and terpenes, among others, represent the most common bioactive phytochemicals, being the latter considered the main group of phytochemicals. In what concerns phenolic compounds, due to their diverse hydroxyl groups, they are described as being highly bioactive, namely in terms of antioxidant properties (Martinez et *al*., 2017).

### II. 2. 1. Phenolic compounds

Phenolic compounds are known to have one or more aromatic rings containing one or more hydroxyl groups in their structure (Liu, 2013). One of the functions that has been described for the phenolic compounds is their influence on the germination of the seeds of other plants (Raimundo *et al*., 2018). There is also a strong connection and positive association between the antioxidant activity and the phenolic content. In fact, phenolic compounds are the most important class of natural antioxidants (H et al., 2013)

According to (Martinez et *al*., 2017), they can be classified into 3 major groups, as shown in Figure 2.

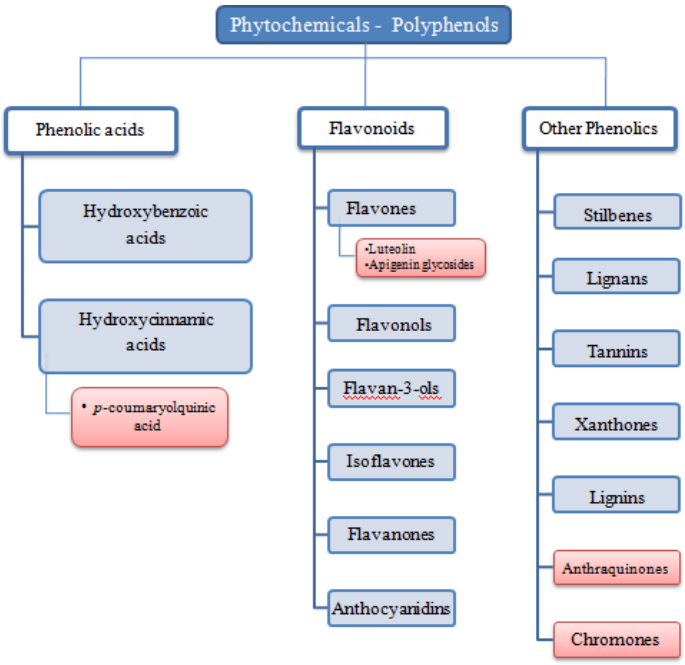


Figure 1. Classification of phenolic compounds (Martinez et *al*., 2017).

Regarding the extraction of these bioactive molecules present in *C. ladanifer*, it has been described that water and ethanol (0.4 kg of biomass in a semi-pilot Soxhlet system (2 L) using water (96 h, 0.35 cycle/h) and ethanol (60 h, 1 cycle/h)) allows the obtention of rutin (Alves-Ferreira, Duarte, Lourenço, et al., 2019) and other flavonoids such as kaempferol derivatives (3-methyl-kaempferol, 3,4-dimethyl-kaempferol, 3,7-dimethyl-kaempferol and 3,7,4'-trimethyl-kaempferol), apigenin and its derivatives 4'-methyl-apigenin, 7-methyl-apigenin and 7,4'-dimethyl-apigenin, which have been identified in the leaf resin (Chaves et al., 1998). In addition, gallic and ellagic acids have also been described (Alves-Ferreira, Duarte, Lourenço, et al., 2019). The biomass fractions have been described to present chemical differences primarily related to extractives. For example, it is possible to have a low extractives content in stems (10.8% evaluated as total phenolics, condensed tannins and flavonoids) compared with that found in the leaves 53.7%. As well as for lignin it is possible to achieve a 21.1% extraction yield in stems and 15.4% in leaves (Alves-Ferreira et *al*., 2020).

The phenolic extract of *C. ladanifer* fresh leaves shows the presence of flavonoids (4.15 mg/g extract), like flavonols, flavones and catechins, phenolic acids and derivatives (3.96 mg/g extract) and ellagic acid derivatives (30.34 mg/g extract). In fact, ellagic acid derivatives were the most prevalent group where punicalagin gallate (punicalag derivative attached to gallic acid), was present in the highest quantity ( 15.99±0.02 mg/g extract) (Barros et *al.*, 2013).

In another study, it was reported that the major compounds found in *C. ladanifer* aerial parts according to Tavares et *al*., (2020) are catechins, gallocatechins, gallic acid, salicylic acid, hydroxycinnamic acid derivatives and tannins.

Tannins are assumed as a large proportion of the overall dry matter of *C. ladanifer* (6.8%) (Barrajón-Catalán et *al*., 2010). According to these authors, four main phenolic compounds were found in the aqueous extract obtained from *C. ladanifer* fresh aerial parts which are gallic acid (0.242+-0.004%, w/w), gallagic acid, ellagic acid and punicalagin isomers, with those three last ellagitannins together accounting for 3.50±0.02% (w/w).

As mentioned, the total phenolic contents can change across various parts of the plant. In fact, the hydromethanolic extract (50:50 v/v) extract from the flowers of *C. ladanifer* exhibited higher phenol content (182.41 mg ferulic acid (FA)/g) than the hydromethanolic extracts of stems (33.77 mg FA/g), leaves (53.49 mg FA/g) and fruits (110.01 mg FA/g) (Zidane et al., 2013). In another study, Andrade et *al*. (2009) reported higher values of the total phenol content, namely 255.19 and 334.46 mg GAE (Gallic acid equivalent)/g for the ethanolic and acetone extracts, respectively, of leaf from *C. ladanifer*.

Important variations between various parts of the plant (flowers, stems, leaves and fruits) were also noticed for the total flavonoid content. The highest content (83.94 quercetin equivalents (QE) mg/g dry weight) was signaled in the ethanolic extract of the fruits from *C. ladanifer* while the lowest was detected in the acetonitrile extract from the stem (3.56 QE mg/g dry weight) (Zidane et *al.*, 2013).

### II. 2. 2. Volatile compounds

Volatile compounds are low molecular weight molecules (ranging from 50 to 200 Da) that have high vapor pressure and mild hydrophilicity (Gressler et *al*., 2011). They are emitted by plants during the developmental phases and as response to different biotic and abiotic stresses (S. Tiwari et al., 2020). Among the many existing volatile compounds, alcohols, esters, acids, terpenes, alkanes and alkenes, can be found in several plants (Kesselmeier & Staudt, 1999).

Isoprenoids are quantitively the most significant compounds present in *C. ladanifer* (Yáñez-Serrano et *al.,* 2018) with monoterpenes and sesquiterpenes dominating the volatile profile. Among monoterpenes, the majority are monoterpene hydrocarbons, ketones, aldehydes and esters (Morales-Soto et *al.,* 2015). Nevertheless, other non-terpene volatile compounds are also present such as alcohols, aldehydes and ketones (Maccioni et *al*, 2007). According to (Bouamama et al., 2006), camphor (43.86%) and α-pinene (17.07%) are the most abundant

compounds of the essential oil obtained from the aerial parts of *C. ladanifer* grown in Spain and extracted using a headspace solid-phase microextraction system. On the other hand, (Teixeira et al., 2007) reported α-pinene, linalool, 2,2,6-trimethylcyclohexanone, borneol, α-terpineol, geraniol, citronellol, geranyl acetate eugenol, and bornyl as C. *ladanifer* essential oil constituents.

In the work of Greche et al. (2009), sixty-eight volatile compounds were detected and quantified in the essential oil extracted from *C. ladanifer* leaves, being the major groups the monoterpene hydrocarbons (22.3%) where the principal components were camphene (12.1%), tricyclene (2.2%), α-pinene (5.0%), and sesquiterpene hydrocarbons (5.8%), with δ-cadinene (2.3%) being the major component of this group.

### II. 2. 3. Other compounds

There are some other important phytochemicals that are present in *C. ladanifer* such as polyunsaturated fatty acids, vitamins and reducing sugars, including polyunsaturated fatty acids from the omega-3 and omega-6 families, that have many beneficial health properties (Guimarães et *al*., 2009). In fact, it has been reported that the presence of α-linolenic acid, a polyunsaturated omega-3 fatty acid, is associated with lower risk of cardiovascular disease (Connor, 2000). Omega-3 fatty acids have also an effect on the brain by avoiding some disorders like Alzheimer's disease and are important for fetal and infant human development. Reducing sugars and vitamins could be efficient in diseases linked to oxidative stress, being also interesting in cosmetics or in dermatological applications (Guimarães et *al*., 2009).

## II. 3. Bioactivity Studies

### II. 3.1. Antimicrobial activity

In general, essential oils are considered as strong antimicrobial agents against several foodborne bacteria and fungi (Contini et *al*., 2020). Different extracts obtained from *C. ladanifer* (e.g., aqueous, hydroalcoholic, acetone:water) demonstrated antimicrobial activity against Gram-positive bacteria such as *Staphylococcus aureus*, Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*, fungi such as *Botrytis cinerea* and yeasts such as *Candida tropicalis* (Raimundo *et al*., 2018).

According to Barrajón-Catalán et *al*. (2010), the antibacterial activity of *C. ladanifer* leaves aqueous extracts was tested using the microdilution method to evaluate the minimum inhibitory

concentration (MIC), against *S. aureus* as model for Gram-positive bacteria and *E. coli* as model for Gram-negative bacteria. Values of $MIC_{50}$ were reported as the antibacterial agent concentration that inhibited 50% of bacterial growth. The results showed that *C. ladanifer* extract was more active against Gram-positive bacteria.

Similarly, in the work of Benayad et *al*., (2013) the antibacterial activity of *C. ladanifer* leaves aqueous extracts was evaluated using the agar diffusion method and the microtitration technique, the latter to establish the MIC values. This work showed that the essential oil of *C. ladanifer* revealed a good antibacterial activity against *Yersinia enterocolitica* and *S. aureus* with MIC values of 62.5 µg/mL and 250 µg/mL respectively and growth inhibition zones of 25 mm for *Y. enterocolitica* and 27 mm for *S. aureus*. Also according to these authors, the highest inhibition zone was identified for methanolic extract towards *Staphylococcus epidermis* (23 mm) and for aqueous extract towards *S. epidermis* (20 mm), *Acinetobacter baumannii* (20 mm) and *S. aureus* (20 mm).

In the work of (Tavares, Martins, Faleiro, et al., 2020), the essential oil obtained from *C. ladanifer* by hydrodistillation also demonstrated antimicrobial activity by using the agar disc diffusion technique, namely against *E. coli* DSM 1077 and *S. aureus* ATCC 6358 obtaining a growth inhibition zone of 10 and 11 mm, respectively.

### II.3.2. Antioxidant activity

In recent years, many sources of antioxidants of plant origin have been studied (Singh et *al*., 2005). Among these, several aromatic plants and spices have been known to be effective in delaying the peroxidation process of lipids (Kulisic et *al*., 2004).

Methanolic and ethanolic extracts from leaves of *C. ladanifer* showed a good antioxidant strength due to their contents in flavonoids, phenolic acids and tannins. Moreover, other antioxidant compounds such as tocopherols, ascorbic acid and reducing sugars were also contributers for its antioxidant properties (Raimundo *et al*., 2018)

The antioxidant potential of *C. ladanifer* extracts prepared with different solvents (ethanol, water, methanol, acetonitrile, ethanol:water (50:50 v/v), methanol:water (50:50 v/v)) as well as that of the essential oil was evaluated by using the 1,1-diphenyl-1-picrylhydrazyl-hydrate (DPPH) free radical scavenging method. A high antioxidant activity was indicated by a high percentage inhibition value. When compared to essential oils from the leaf of *C. libanotis*, all extracts from the different plant parts of *C. ladanifer* showed higher DPPH radical scavenging

potential. In fact, methanolic extracts revealed an oxidative inhibition of 97.8% for the leaf, 97.3 % for stems, 96.1% for flowers and 95.9%) for the fruits (H et *al*., 2013).

Barrajón-Catalán et *al*. (2010) used several methods to thoroughly assess the antioxidant activity of *C. ladanifer* aqueous extracts, namely the trolox equivalent antioxidant capacity (TEAC), ferric-reducing ability power (FRAP), radical-scavenging capacity by thiobarbituric acid-reactive substances (TBARS) and oxygen radical absorbance capacity assay (ORAC). The authors reported that the TEAC value obtained for *C. ladanifer* (35.85±1.25 mmol trolox equivalent/100 g dry weight) was close to other values recorded for traditional plants for medicinal use. Moreover, the ORAC value obtained (3329.0 ± 182 µmol trolox equivalent /g dry weight) could reveal useful applications for the aqueous extract of *C. ladanifer* as a preventive agent against peroxyl radicals that cause the oxidation of lipids in biological systems and food.

### II.3.3. Cytotoxicity

Over the years, advantageous biological activities of essential oils and their components have been recognized, including towards human health benefits (Contini et *al.*, 2020).

The cytotoxic activity of *C. ladanifer* has been confirmed for both the labdanum resin and for the aqueous and ethanolic extracts due to the presence of phenolic compounds (Raimundo *et al*., 2018).

Barrajón-Catalán et *al*. (2010) evaluated the cytotoxic potential of *Cistus* aqueous extracts against a large set of human cancer cell lines, including colon, breast and pancreatic cancer cells, by using the MTT assay, which is a fast, flexible and quantitative colorimetric assay, relying on tetrazolium salt MTT that testes living cells (Mosmann et *al*., 1983). The results showed that the M220 pancreatic cancer cells were highly susceptible, with a 50% cytotoxic concentration ($CC_{50}$) value of 0.49 mg/ml, while MCF7/HER2 and JIMT-1, were the most susceptible breast cancer cells for *Cistus* extract, with $CC_{50}$ values ranging between 1.6-2 mg/ml and 0.5-1 mg/ml, respectivelly (Barrajón-Catalán et *al*., 2010).

### II.3.4. Anti-inflammatory and anti-nociceptive activities

Inflammation is a form of protection shown by organisms against noxious stimuli. Further, the rage of variables and mediators of inflammation can be inhibited with anti-inflammatory agents such as expression of adhesion molecules, growth factors and cytokines (Chandrakanthan et *al*., 2020).

The central analgesic activity from the aqueous extract of *C. ladanifer* was investigated by the hot plate process while the acute anti-inflammatory activity was examined by rat paw edema caused by 0.5% subplantear injection of carrageenan in the right hind paw (El Youbi et al., 2016). Pretreatment of rats with intraperitoneal injection of *C. ladanifer* aqueous extracts at various doses (150, 175 and 200 mg/kg body weight (b.w.)) showed that the edema paw inflammation after injection of carrageenan decreased significantly at all doses. The percentage of edema inhibition was 66.67%, 67.65% and 77.78% for the doses of 150, 175 and 200 mg/kg b.w., respectively. Moreover, in thermal-induced pain models, this aqueous extract showed a major analgesic effect, illustrated by a substantial reduction in the pain score at all doses after administration of the aqueous extract. In this situation, the nociception prevention result after the administration of *C. ladanifer* aqueous extract were 70.3%, 74.55% and 93.33% for the doses of 150, 175 and 200 mg/kg b.w., respectively (El Youbi et *al*., 2016).

In another study, the albumin denaturation assay was used to test the anti-inflammatory activity of the hydrolate provided from steam distillation of *C. ladanifer* (Tavares, Martins, Faleiro, et al., 2020). The results demonstrated a potent anti-inflammatory activity of *C. ladanifer* hydrolate around 94% of inflammatory inhibition capacity.

Overall, *C. ladanifer* aqueous extract leaves were reported to be able of inhibiting edema (which is a buildup of fluid in the body's tissue) in rats. In terms of pain relief, nociceptive reaction responses were considerably affected indicating the high potential of aqueous extracts (Raimundo et al., 2018).

### II.3.5. Insecticidal activity

Essential oils are also investigated as potent candidates for developing natural insect control agents (Chen et *al.*, 2021)**.**

*C. ladanifer* branches are used to catch house flies that get stuck in the sticky leaves attracted by their strong scent. Moreover, the presence of flavonoids in leaves has an impact on the plasticity of insects skeletal muscle fibers (Raimundo *et al*., 2018).

The effect of the methanol extract of *C. ladanifer* aerial parts on *Plodia interpunctella*, a common cosmopolitan pest of added-value products and stored grains, with a large distribution that causes economic losses by reducing the quality of food, was shown since it affected the postembryonic growth parameters. In fact, *C. ladanifer* extract caused a larval weight reduction of up to 18% after 8 days of treatment in addition to the complete inhibition of adult emergence and the decrease in pupation (Bouayad et *al*., 2013).

## II.4. Industrial applications

Overall, *C. ladanifer* has been reported to have several different uses, including a diversity of industrial applications, with the most relevant being described, as follows.

- **Perfumery**

Many members of the Cistaceae family are very fragrant and sweet-smelling, therefore being highly valued by the perfume industry, as well as for ornamental purposes(Barrajón-Catalán et *al*., 2010). That includes *Cistus ladaniferi,* which is considered as the most common species of this family used in the perfume and cosmetic industries (Greche et *al*., 2009).

In particular, *C. ladanifer* has a great interest in the perfume industry. In fact, labdanum is one of the key components of some high-quality perfumes, especially in men's fragrances. The odour of fragrances derived from rockrose can differ depending on the kind of compounds present, including herbal, floral/herbal, minty/fresh, citrus and balsamic odours. Comparing the composition in amber, labdanum seems to be richer than the essential oil. Due to the presence of diterpene compounds all the extracts of labdanum have a fragrance of sweet rich and long lasting odour notes (Raimundo *et al*., 2018).

*C. ladanifer* contains compounds such as linalool, α-terpineol, borneol, citronellol, eugenol, geraniol, and bornyl acetate that are considered as high-value substances for cosmetics and perfume industry and can be used in various personal care items and fragrances (Teixeira et *al*., 2007).

According to Tavares et al. (2020), the essential oil obtained from *C. ladanifer* has been linked to multiple biological activities and is an added-value biomass product that can be easily used in the perfume and cosmetic industry or as bioblocks for various other industries.

- **Phytoremediation**

*C. ladanifer* bioextracts are known to have a potential role in the phytoremediation of trace element contaminated soils by phytoextraction or by phytostabilization, which explains the fact that this plant is widely found on soils rich in metals (Raimundo et *al*., 2018).

Most studies concentrate on six significant trace elements, namely Cu, As, Mn, Pb, Zn and Ni. *C. ladanifer* (bioaccumulation factors < 1) has been reported to serve as an excluder for Cu, As, Pb and Ni. The concentrations of these components in the aerial parts of the plant are therefore low, despite the high total concentrations in the soil. In the other hand, the populations of *C.*

*ladanifer* developing on mine tailings are Mn accumulators. In fact, this element's foliar concentration can exceed 7000 µg/g which is considered as a very high value close to the hyperaccumulation threshold (10,000 µg/g). This shrub is also a Zn accumulator despite its low foliar concentration that is around 200-300 µg/g (van der Ent et *al*., 2013).

(Simões et al., 2009) also highlighted the beneficial impact of *C. ladanifer*, confirming the increase in soil quality and the likelihood of encouraging vegetation regeneration by easing the invasion of more demanding species.

- **Food**

It is claimed that flowers and hard seeds of *C. ladanifer* are edible (Tardío et *al*. 2006). These seeds are composed of phospholipids (5 %), steryl esters, free sterols, acyl steryl glycosides and steryl glycosides (together 18 %) and triacylglycerols (75 %) (Krollmann & Gülz, 1983). While used in low doses to prevent anxiety, insomnia, gastritis and ulcers, in coffee, tea and other infusions, the use of labdanum and solvents-obtained extracts is limited because of their hepatotoxic, nephrotoxicity and neurotoxic reported effects (Barrajón-Catalán et al., 2010)(Delgado et al., 2001). Also, in western Spain, one of the most commonly cited effect is the raw consumption of a sugary exudate which is known as 'miel de jara' or 'màngala'. The soft sections of *C. ladanifer* are used in the traditional Portuguese gastronomy for seasoning rabbit-filled dishes (Tardío et *al*. 2006). On another hand, due to its exceptional nutritional value, medicinal and nutraceutical potential, bee pollen has been eaten for centuries since studies have not found any harmful compounds in *C. ladanifer* pollen. This bee pollen has been reported to contain kaempferol, myricetin and flavonoid glycosides of quercetin that are linked to several biological activities such as antioxidant, anti-inflammatory, antibacterial, androgenic and anti-osteoporosis effects (Raimundo *et al.*, 2018).

- **Feed**

Aerial parts of *C. ladanifer* may be a source of bioactive compounds, such as condensed tannins, terpenes, flavonoids and phenolic acids, in ruminant diets. It can also play a significant role in the process of welfare and wellbeing of animal performance and promote product quality because of the presence of phenolic acids, flavonoids and condensed tannins (Guerreiro et *al*., 2016).

According to (Jerónimo et al., 2010)(Jerónimo et al., 2012), *C. ladanifer* increases the antioxidant activity of meat in lamb diets. In addition, the inclusion of *C. ladanifer* can enhance

the nutritive value of the lipid fraction of lamb meat in oil-supplemented diets, raising its content in rumenic acid, which is a conjugated linoleic acid isomer that has demonstrated numerous beneficial health properties.

*C. ladanifer* condensed tannin extracts have been used in ruminant animals to increase the digestive use of feed proteins. In fact, ruminant animals of high production need diets with high levels of undegradable rumen protein, normally known as "bypass protein," that are not completely degraded in the rumen, but partially digested and absorbed in the small intestine, providing the animal with the necessary amino acids to fulfil its needs. Studies have shown that using a tannin extract from *C. ladanifer* to preserve soybean protein, a decrease in ruminal degradation was observed, enhancing the flow of feed protein into the post ruminal compartments where it is divided into amino acids and eventually digested and used by the animal (Dentinho et al., 2007) (Dentinho et al., 2014).

- **Biofuels and other applications**

The potential for using *C. ladanifer* in biofuel production through ethanolic fermentation in bioconversion studies has been reported to be very high. A critical step in the bioethanol production process is the pre-treatment of lignocellulosic biomass before polysaccharide hydrolysis.

Pyrolysis-treated *C. ladanifer* may return 2106kcal ha-1 (with one-year-old plants). The resulting bio-oil from slow pyrolysis of *C. ladanifer* may be also used in pharmaceutics and cosmetics industries as a renewable fuel whose calorific value is similar to that of petroleum fractions. This product release to the atmosphere less polluting gases because of it lack in sulfur (Raimundo *et al.*, 2018).

Recently, (Carrión-Prieto et al., 2017) showed that the use of *C. ladanifer* in rural district heating, in the form of pellets, defines the criteria for harvesting (limited to the most robust specimens and without leaves) in order to ensure that the requirements of current European biofuel standards are sufficient.

Other studies show that solid residues derived from *C. ladanifer* distilleries can be used as feedstock for the production of bio-product solutions and biofuel in bio-refineries (Alves-Ferreira, Duarte, Fernandes, et al., 2019).

## III. Objectives

As mentioned, the essential oil and/or extracts obtained from *C. ladanifer* are interesting products for the cosmetic and perfume industries and can also be potentially used also in the food and pharmaceutical industries. Moreover, this species is also known for its ability to grow on marginal lands and can therefore be a valuable source of biomass for bio-based industries. Thus, this work aims at valorising *C. ladanifer* biomass as a source of compounds for industrial application. For that purpose, the work will focus on the extraction of its essential oil and also on the recovery of bioactive molecules still present in the residue of the hydrodistillation. In what concerns the essential oil, different extraction methodologies, namely hydrodestillation in Clevenger system, ultrasound-assisted extraction (UAE) and microwave extraction (MAE) will be assayed aiming for the optimization and increased yield at laboratory scale. Besides extraction yield, the chemical composition of the obtained essential oils will be determined by GC-MS. Finally, the generated distillation residues will be evaluated for their composition in phenolic compounds, within a biorefinery concept.

## IV. Materials and Methods

### IV.1. Sample collection

The aerial parts of *Cistus ladanifer* were collected from the Parque de Montesinho (Bragança, Portugal) during the month of May, at the flowering stage. The plant was either used in the days after its collection or dried in the dark at ambient temperature and stored in the lab until being used in the experiments. In both cases, the leaves and buds were manually separated. In addition, at the same time of the collection of the branches containing the leaves and buds, the flowers were also separately collected.

Figure 2. Aerial parts of *Cistus ladanifer* containing leaves and buds.

### IV.2. Essential oil and concretes

#### IV.2.1. Hydrodistillation

The essential oil of the leaves and the buds was extracted by hydrodistillation in a Clevenger apparatus (Fig. 4) at the boiling temperature of water and atmospheric pressure. The extraction process was performed using 100g of sample, and a solid (g) to solvent (mL) ratio equal to 1:20 during 3 hours in a 4L balloon. In this type of extraction the fresh plant was used.

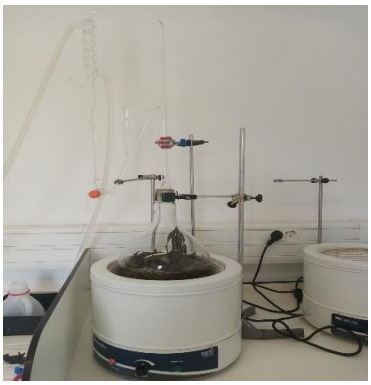

Figure 3. Clevenger apparatus

### IV.2.2. Soxhlet extraction

For each sample of leaves and buds, two extractions were preformed, namely an extraction with ethanol and another using hexane. In both, approximately 3 g of grinded plant material was extracted in a Soxhlet apparatus during 6 h at the boiling temperature of each corresponding solvent. The extracts were then evaporated in a rotary evaporator (50°C, 100 rpm) to obtain the Soxhlet ethanolic and hexane extract (also know as concretes). The extractions were performed at least in duplicate.

Figure 4. Extraction of concretes on soxhlet apparatus

### IV.2.3. Microwave assisted extraction (MAE)

After that the samples (leaves and buds) were ground in a blender, the extraction was performed on a microwave equipment Model NuWav-Uno (power consumption 1200 watts(Max.), voltage 220 vac/50 Hz , dimensions 550 mm*360 mm* 495 mm) (Fig. 6) using

the conditions presented on Table 1. The extraction was performed using 5 g of sample and a solid (g) to solvent (mL) ratio equal to 1:20. After the extraction process the extracts were filtered and evaporated in a rotary evaporator (50°C, 100 rpm). The extractions were performed at least in duplicate.

Table 1. Conditions used for obtaining *C. ladanifer* concretes using MAE.

| | **Ethanol** | **Hexane** |
|---|---|---|
| **Time (min)** | 15 | 15 |
| **Temperature (°C)** | 80 | 60 |
| **Power (W)** | 400 | 500 |

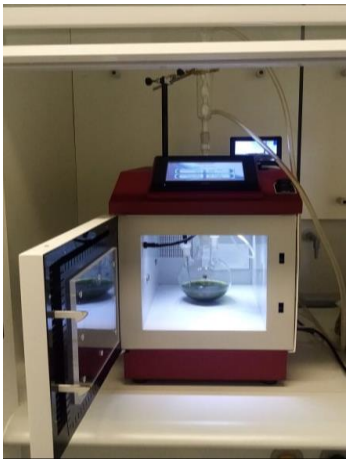

Figure 5. Microwave equipment used in the extractions

### IV.2.4. Ultrasound assisted extraction (UAE)

The plant material was dried at room temperature in the shade and ground prior to extraction. Approximately 5 g of sample was mixed in 100 mL of solvent (ethanol or hexane) and subjected to the ultrasound-assisted extraction using a Qsonica Q500 type ultrasound probe (50 kHz, 500 W maximum power, 203 mm × 387 mm × 216 mm internal dimensions) during 10 min. The crude extracts were then filtered and evaporated in a rotary evaporator (50°C, 100 rpm) to obtain the ultrasound-assisted ethanolic and hexane extracts (concretes). The conditions used in the extractions were the following:

- maximum volume: 150 mL
- Time: 10 min, comprising cycles of 30s and 10s of pause to avoid overheating.

- Power: 375W corresponding to 75% of the power (500W) .
- Ice bath

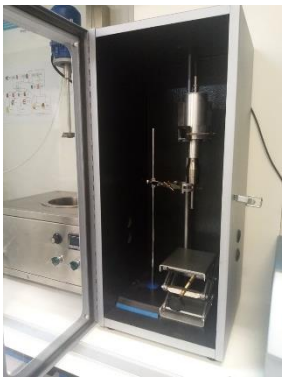

Figure 6. Ultrasound equipment used in the extractions

### IV.2.5. Gas-chromatography coupled to mass spectrometry

The obtained essential oils and concretes were analysed by gas chromatography coupled to mass spectrometry detection (GC-MS) using a GC-2010 Plus (Shimadzu) system equipped with an AOC-20iPlus (Shimadzu) automatic injector and an SH-RXi-5ms (30 m x 0.25 mm x 0.25 μm; Shimadzu, USA) column. The essential oils were diluted in hexane while the concretes were diluted in the respective solvent (50 mg/mL) and then filtered using a filter (with a pore size equal to 22μm and 25 diameter). The volume of the injected sample was 1 μL. Helium was used as the carrier gas. The identification of compounds was based on the comparison of the obtained mass spectra with those of the NIST17 mass spectra library and linear retention indexes (LRI) and comparison with published data. The LRI were calculated from the retention times of an n-alkane series analyzed under identical conditions. Compounds were quantified as area percentages of total volatiles using the relative values directly obtained from peak total ion current (TIC).

## VI.3. Bioactive proprieties

### VI.3.1. Antioxidant activity

Different antioxidant assays were performed in order to deeply characterize the antioxidant potential of all the extracts, which were designated as follows: leaves extracted with ethanol using Soxhlet, LES; buds extracted with ethanol using Soxhlet,BES; leaves extracted with hexane using Soxhlet,LHS; buds extracted with hexane using Soxhlet, BHS; leaves extracted with ethanol using MAE, LEMW; buds extracted with ethanol using MAE, BEMW;

leaves extracted with hexane using MAE, LHMW; buds extracted with hexane using MAE, BHMW; leaves extracted with ethanol using ultrasounds, LEU; buds extracted with ethanol using ultrasounds, BEU; leaves extracted with hexane using ultrasounds, LHU; buds extracted with hexane using ultrasounds, BHU.

The antioxidant activity was evaluated by assessing the scavenging capacity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and reducing power assay. Those two assays were performed using an ELX800 microplate reader (Bio-Tek Instruments, Inc.; Winooski, VT, USA) (Fig. 8).

Figure 7. Microplate readar used for assessing antioxidant activity

- **DPPH assay**

In this assay, 3 µL of each of the extract solutions was mixed in a 96-well plate containing 30 µL of ethanol, after doing the dilutions 270 µL of methanolic DPPH solution ($6*10^{-5}$ M) was added. Then the plate is being read at 515 nm after staying for 60 min in the dark

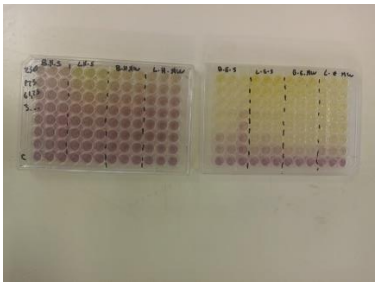

Figure8. Plates of the DPPH assay

- **Reducing power**

For each concentration of the extracts (2.5mg/mL , 200 mg/mL ) 0.5 mL were mixed with sodium phosphate (200 mmol/L, pH 6.6, 0.5 mL) and 0.5 mL of potassium ferricyanide (1% (w/v), then the mixtures were incubated at 50 °C for 20 min in a bath. After that, 0.5 mL of trichloroacetic acid (10% (w/v)) was added to stop the reaction. The mixture (0.6 mL) was transferred into a 48-well plate containing 0.6 mL of distilled water and finally 0.12 mL of ferric chloride (0.1% w/v) was added. The reducing power was evaluated by measuring the capacity of the extract to reduce $Fe^{3+}$ to $Fe^{2+}$ by measuring the absorbance at 690 nm.

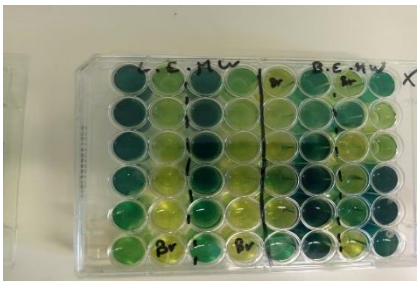

Figure 9. Plate of the reducing power assay

### IV.3.2. Antibacterial activity

- **Microorganisms**

The bacteria used in this work were clinical isolates and ATCC strains. The clinical isolades were obtained from patients hospitalized in various departments at the Northeastern local health unit (Bragança, Portugal) and Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Those comprised five Gram-negative bacteria, namely *Escherichia coli* (isolated from urine), *Proteus mirabilis* (isolated from wound exudate), *Klebsiella pneumoniae* (isolated from urine), *Pseudomonas aeruginosa* (isolated from expectoration) and *Morganella morganii* (isolated from urine), and three Gram-positive bacteria, namely *Enterococcus faecalis* (isolated from urine), *Listeria monocytogenes* (isolated from cerebrospinal fluid), and methicillin-resistant *Staphylococcus aureus* (MRSA) (isolated from expectoration). The ATCC strains comprised several bacteria frequently associated to food borne diseases and included five Gram-negative bacteria, namely *Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella* enterica subsp. enterica (ATCC 13076), *Yersinia enterocolitica* (ATCC 8610) and three Gram-positive bacteria, namely

*Bacillus cereus* (ATCC 11778), *L. monocytogenes* (ATCC 19111) and *S. aureus* (ATCC 25923). ATCC strains were purchased at Frilabo, Porto, Portugal. All these microorganisms were incubated at 37 ºC in an appropriate fresh medium for 24 h before analysis to maintain the exponential growth phase.

- **Determination of Minimum Inhibitory (MIC) and Minimum Bactericidal concentrations (MBC)**

The MIC determinations for all bacteria were conducted using the microdilution method and the iodonitrotetrazolium dye allowing a colorimetric measurement according to described by Pires et al, 2018 with some modifications. Sterilized Muller-Hinton broth (MHB) added with 0.5% Tween 80 was used as culture media for the assay. The inoculum was prepared by adding 30 mL of MHB broth and 200 µL of inoculum (standardized at $1.5\times10^6$ Colony Forming Units (CFU) /mL) in a sterile Schott bottle. The sample dilutions (2.5%, 1.25%, 0.625%, 0.313%, 0.156%, 0.078%, 0.03% and 0.01%, v/v) were prepared by adding 190 µL of medium MHB with Tween 80 and 10 µL of sample (EO) in the first well of a 96-well microplate in duplicate. The remaining wells were added with 90 µl of MHB medium with Tween 80. Then the samples were serially diluted to obtain the concentration ranges (2.5% to 0.01%). Finally 10 µL of inoculum (standardized at $1.5\times10^6$ Colony Forming Unit (CFU) /mL) was added to the well. Two negative controls were prepared: MHB with Tween 80 and another one with the extract. Two positive controls were prepared with MHB with Tween 80 and each inoculum and one with culture medium, antibiotics and bacteria. Ampicillin and Streptomicin were used for all bacteria tested and Meticilin was also used for *S. aureus*. The microplates were incubated at 37°C for 24 h. The MIC was detected following the addition (40 µl) of 0.2 mg/mL p-iodonitrotetrazolium chloride (INT) and incubation at 37°C for 30 min. MIC was defined as the lowest concentration that inhibits the visible bacterial growth determinate by change the coloration from yellow to pink if the microorganisms are viable. For the determination of MBC, 10 µL of liquid from each well that showed no change in colour was plated on solid medium, Blood agar (7% sheep blood) and incubated at 37°C for 24 h. . MBC was defined as the lowest concentration required to kill bacteria and corresponded to the lowest concentration that yielded no growth determine the MBC.

### III.3.3. Antifungal activity

The antifungal activity was performed according to described by Heleno et al., 2013. With some modifications.

*Aspergillus fumiga*tus (ATCC 204305), *Aspergillus brasiliensis* (ATCC 16404) were used in the assays. The organisms were obtained from Frilabo, Porto, Portugal. The micromycetes were maintained on malt agar and the cultures stored at 4 °C and were further placed in new medium and incubated at 25ºC for 72h. In order to investigate the antifungal activity, the fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately $1.0 \times 10^5$ in a final volume of 100 µL per well. The samples were first of all mixed with medium MHB with Tween 80. Afterwards, 100 µL of each sample was added in the first well (96-well microplate) in duplicate with 90 µL oIn the remaining wells, 90 µL of medium MEB were placed. Then the samples were serially diluted to obtain the concentration ranges (2.5% to 0.01%). Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microplate. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentration (MFC) was determined by serial subcultivation of a 2 µL of tested compounds dissolved in medium and inoculated for 72 h, into microplates containing 100 µL of MEB per well and further incubation 72 h at 26 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. Commercial fungicide ketoconazole (Frilabo, Porto, Portugal), was used as positive control.

### IV.4. Response surface methodology

The optimization protocol was performed using Design Expert 11 (Stat-Ease, Minneapolis, MN, USA), and relied on the Response Surface Methodology using Box-Behnken models for the design of experiments followed by an optimization of the maximization of the response as we as the desirability function (optimal conditions for all responses) for the buds and leaves.

## V- Results and discussion

### V.1. Essential oils

Respecting the essential oil extracted from the leaves of rockrose 55 compounds, accounting for 67.21% of total compounds, were identified using gas chromatography coupled to mass spectrometry (GC/MS) as shown in Table 2. Most of the compounds identified belong to different groups of terpene and terpenoid compounds, namely monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons and oxygenated sesquiterpenes. *Cistus ladanifer* produced an essential oil rich in oxygenated compounds, with 9 oxygenated sesquiterpenes that accounted for 40.14% and 20 oxygenated monoterpenes that represented 17.73%. The third most abundant class of compounds found in the essential oil were sesquiterpenes hydrocarbons with eight identified compounds, accounting for 3.26%.

In the essential oil extracted from the buds 51 compounds were identified accounting for 57.99% of the essential oil. From the identified compounds, 8 were oxygenated sesquiterpenes that accounted for 30.08% and 19 were oxygenated monoterpenes corresponding to 17.36%. Compared with the leaves, a lower % was found for the group of oxygenated sesquiterpenes while a slightly higher % was obtained for the group of monoterpene hydrocarbons (3.25%) The groups of sesquiterpenes (3.12%) and oxygenated monterpenes presented similar amounts in both samples. The chemical composition of the hydrodistilled oil from leaves and buds showed to be different, both qualitatively and quantitatively. The major compounds in the leaves were viridiflorol (13.2%), spathulenol (9.7%), 2,2,6-trimethylciclohexanone (5.2%), ledol (4.1%) and bornyl acetate (2.1%) while on the buds were viridiflorol (25.2%), ledol (10.4%), transpinocarveol (3.2%) bornyl acetate (3.0%) and 2,2,6-trimethylciclohexanone (2.4%). As can be observed in Table 2, curiously the composition of the two samples in the aromadendrane compounds spathulenol and ledol was distinct, while the leaves present a high amount of the last, which is only found in minute amounts (0.8%) in the buds, the former showed a much higher content in the buds (10.4% vs 4.1%). According to the literature, anti-inflammatory (Raina Maclnture, 2020) and antifungal (Gijsen et al., 1995) activities has been attributed to spathulenol. Ledol has been also described has having antifungal activity (Duran-Pena et al., 2015), however it has also been associated with toxic effects and it can be responsible for adverse reactions such as dizziness, nausea and vomiting (Jesionek et al., 2018). The essential oil of *Ledum palustre* that presents ledol as a major constituent, has been used as a medicine against various diseases and to provoke abortion. However, due to is toxicity, on

digestion ledol can cause convulsions, followed by paralysis (Gijsen et al., 1995). Therefore, and despite the essential oil not being intended for oral ingestion, based in this aspect it could be interesting to collect the plant before the flowering buds stage when the purpose is to obtain *Cistus ladanifer* essential oil. On the other hand, a higher content of ledol's isomer viridiflorol (Figure 10), which has been demonstrated to exhibited biological activities, such as anti-mycobacterial, anti-inflammatory and antioxidant activity (Trevizan et al., 2016) was observed in the buds compared to the leaves.

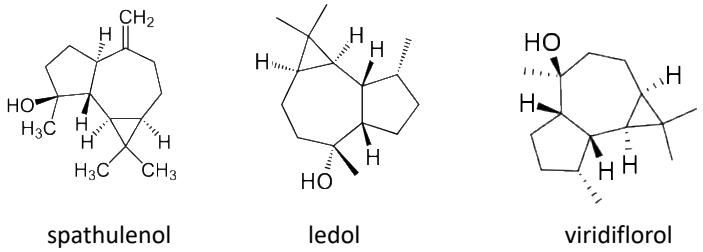


Figure 10. Chemical structures of different compounds with aromadendrane structure found in the essential oils of *C. ladanifer.*

The composition of *C. ladanifer* essential oil previously reported in the literature is in general very distinct from the one obtained in this study. This can be attributed not only to the different edaphoclimatic conditions associated with the collected specimens, to different plant parts and extraction methods, but also to the possible existence of different chemotypes. Moreover, in the present work the essential oil was extracted from the fresh plant while in several works the plant is used in the dried form. Mariotti et al. (1997) analyzed the essential oil obtained by industrial hydrodisttilation of a bulk sample of stems and leaves and reported α-pinene as being the major compound (39%) followed by viridiflorol (11.2%), ledol (3.3%) and bornyl acetate (3.1%). The same authors also evaluated 20 individual samples obtained by Clevenger hydrodistillation of stems and leaves and observed the existence of 6 groups that showed different chemical profiles. All of those groups had in common α-pinene as major compound (11.1-47.4%), with the exception of one group comprised of two plants that showed a completely different profile characterized by having viridifloral (20.0-22.6%) as the most abundant constituent, followed by trans-pinocarveol (5.4-8.6%) and ledol (6.4-6.7%). Mrabet et al. cited by Gomes et al. (2005) analyzed the essential oil of two varieties of cistus grown in Morocco and found viridiflorol as the main constituent (12.81-20.68%) and a very low content of α-pinene (0.30-0.37%). The samples herein studied also showed a similar profile, which points to the possible existence of a chemotype characterized by the high levels of viridiflorol and low of α-pinene. This result is in good agreement with that reported by Gomes et al. (2005)

that studied 2 samples collected in the mountainous region of the center-interior of Portugal and also found high contents of viridiflorol (13.6-17.4%). However, the authors reported globulol as second major compound (3.1-5.0%) and a non-identified sequiterpene alcohol (2.7-6.0%) while in the present work globulol was only found in the buds and in low content (0.9%).

The most frequent profile characterized by high levels of α-pinene has also been reported to occur in Portugal. Regino et al. cited by Gomes et al. (2005) studied the composition of cistus grown in Portugal, concluding that despite the variation of composition and yield of cistus oil along the year, the main components were always α-pinene (5.34-25.56%), 2,2,6-trimethylcyclohexanone (4.46-11.88%) and ledol (6.59-13.78%). Simon-Fuentes et al. (1987) also found the main constituents to be monoterpenes, namely α-pinene (35%), camphene (10.3%), p-cymene (4%), bornyl acetate (3.7%), isopinocamphone (3.5%) and pino-carveol (3.4%), also reporting high content of the norterpene 2,2,6-tri-methylcyclohexanone (5.7%). A profile pointing to a chemotype rich in α-pinene was also reported by Morales-Soto et al. (2015) who identified 51 compounds in *C. ladanifer* aerial parts collected from Spain. In this work α-pinene (17.1%), bornyl acetate (15.3%), camphene (13.0%), 2,2,6-trimethyl-cyclohexanone (12.3%), and α-campholenal (4.7%) were the most abundant compounds, but it should be considered that a different extraction method was used, namely headspace solid-phase microextraction. Despite most chemotypes described in the literature have α-pinene or viridiflorol as major compounds others have been reported, such as the essential oil from Moroccan *C. ladanifer* essential oil, which was characterized by the presence of camphene (15.5%), borneol (11.1%), 2,2,6 trimethylciclohexanol (7.3 %), terpineol-4 (6.3 %) and α-pinene (4.2 %) as major compounds (Zidane et al., 2012).

Among the compounds described in Table 2, some have been described as key-odor compounds in the essential oil of *C. ladanifer*. This is the case of viridiflorol (floral); terpinen-4-ol (spicy floral); bornyl acetate and borneol (fresh-camphoraceous), 2,2,6-trimethylcyclohexanone (rock-rose leaf odor), acetophenone (floral aroma) and 6-oxoisoambrox (soft animal amber/ambery character). Other compounds existing in small amounts (in general <1%) described by Weyerstahl et al. (1998) as being olfactory key components for the amber scent in *C. ladanifer* such as 11-nor-driman-8-ol (powerful woody-amber), ambrox (strong dry amber), α-ambrinol (warm animal amber), drimenone (tobacco, amber), 6-acetoxy-nor-drimenone (soft warm, strong amber, woody), oxoambrox (soft animal amber, like ambrox), sclareolide (weak fine amber, woody-resinous tone) and labdenols (weak amber-animal) were not detected in the studied samples.

Overall, the studied samples, both the leaves and buds, showed low values of monoterpenes that frequently degrade easily or diminish with the drying of the plants (Gomes et. Al, 2005) and the present of different floral and amber odorant compounds, which is desirable from an organoleptic point of view.

Table 2. Chemical composition of *Cistus ladanifer* essential oil determined by GC-MS.

| **Compounds** | **Rt(min)** | **Calculated LRI [a]** | **Theoretical LRI [b]** | **Leaves** | **Buds** |
|---|---|---|---|---|---|
| | | | | **mean + SD (%)** | **mean +SD(%)** |
| Tricyclene | 13.49 | 917 | 921 | - | 0.015±0.002 |
| α-pinene | 14.12 | 929 | 932 | 1.735±0.078 | 0.136±0.001 |
| Camphene | 14.86 | 944 | 946 | 0.805±0.007 | 0.1372±0.0003 |
| Thuja-2,4(10)-diene | 15.18 | 950 | 953 | 0.095±0.007 | 0.019±0.001 |
| Benzaldehyde | 15.49 | 956 | 952 | - | 0.022±0.001 |
| 6-Methyl-5-heptene-2-one | 16.97 | 984 | 981 | - | 0.0208±0.0004 |
| α-Terpinene | 18.45 | 1013 | 1014 | | 0.036±0.001 |
| p-cymene | 18.87 | 1021 | 1020 | 0.34±0.014 | 0.16±0.01 |
| Limonene | 19.08 | 1025 | 1024 | 0.13±0.01 | 0.03±0.001 |
| 2,2,6-Trimethylcyclohexanone | 19.34 | 1030 | 1035* | **5.18±0.07** | **2.4±0.1** |
| 2,4,4-Trimethyl-2-cyclohexen-1-ol | 20.36 | 1049 | n.r. | 0.095±0.007 | 0.057±0.005 |
| Acetophenone | 21.04 | 1063 | 1059 | 0.105±0.007 | 0.198±0.01 |
| cis-Linalool oxide | 21.36 | 1069 | 1067 | - | 0.069±0.001 |
| Linalool | 22.79 | 1096 | 1095 | 0.115±0.007 | 0.095±0.007 |
| cis-Rose oxide | 23.34 | 1107 | 1106 | 0.21±0.01 | 0.149±0.003 |
| α-Campholenal | 24.08 | 1122 | n.r. | 1.02±0.07 | 0.766±0.006 |
| trans-Rose oxide | 24.16 | 1124 | 1122 | 1.02±0.07 | - |
| Cosmen | 24.31 | 1127 | 1130* | 0.15±0.01 | 0.107±0.005 |
| Trans-pinocarveol + not identified compound | 24.75 | 1136 | 1136 | 1.00±0.05 | **3.2±0.2** |
| cis-Tagetone | 25.51 | 1151 | 1160 | - | 0.51±0.01 |
| Pinocarvone | 25.91 | 1159 | 1165 | 0.805±0.007 | 0.55±0.01 |
| borneol + *p*-Mentha-1,5-dien-8-ol | 26.13 | 1163 | 1165 | 0.81±0.03 | **1.9±0.1** |
| cis-3-Pinanone | 26.48 | 1171 | 1172 | - | 0.39±0.02 |
| Terpinen-4-ol | 26.66 | 1174 | 1174 | 0.39±0.04 | 0.49±0.04 |
| p-Cymen-8-ol | 27.05 | 1182 | 1179 | 0.395±0.007 | |
| Myrtenal | 27.59 | 1193 | 1194 | 0.53±0.01 | 0.7±0.02 |
| Verbenone | 28.19 | 1205 | 1204 | 0.35±0.04 | 0.157±0.007 |
| trans-Carveol | 28.68 | 1216 | 1215 | 0.375±0.007 | 0.27±0.01 |
| cis-Tagetenone | 29.26 | 1228 | 1226 | 0.44±0.03 | 0.56±0.02 |
| trans-Tagetenone | 29.66 | 1237 | 1235 | - | 0.541±0.03 |
| Carvone | 29.84 | 1241 | 1239 | 0.095±0.007 | - |
| cis-Geraniol | 30.35 | 1251 | 1249 | 0.78±0.04 | - |
| Bornyl acetate | 31.83 | 1283 | 1285 | 2.1±0.1 | **3.0±0.1** |

| | | | | | |
|---|---|---|---|---|---|
| (Z)-Hex-3-enyl (E)-2-methylbut-2-enoate | 33.53 | 1321 | n.r. | - | 0.067±0.007 |
| Myrtenyl acetate | 33.62 | 1321 | 1324 | 0.62±0.06 | 0.059±0.004 |
| Cyclosativene | 35.56 | 1367 | 1369 | 0.48±0.02 | 0.8±0.02 |
| α-Copaene | 35.91 | 1375 | 1374 | - | 0.226±0.004 |
| Geranyl acetate | 36.14 | 1380 | 1379 | 0.27±0.01 | - |
| 4-(4-Methylphenyl)pentanal | 36.97 | 1398 | 1406* | 0.315±0.007 | 0.36±0.003 |
| 9-epi-(E)-Caryophyllene | 39.57 | 1461 | 1461 | 0.45±0.01 | 0.68±0.01 |
| Geranyl propionate | 39.96 | 1470 | 1473* | 0.31±0.01 | - |
| γ-Muurolene | 40.19 | 1476 | 1478 | - | 0.129±0.002 |
| (+)-Ledene | 40.98 | 1495 | 1496 | 0.33±0.01 | 0.694±0.03 |
| 1,1,4,5,6-Pentamethyl-2,3-dihydro-1H-indene | 41.88 | 1517 | n.r. | 0.27±0.01 | 0.352±0.005 |
| δ-Cadinene | 42.09 | 1522 | 1523 | 0.275±0.007 | 0.82±0.07 |
| α-Calacorene | 42.86 | 1542 | 1544 | 0.49±0.04 | 0.371±0.002 |
| palustrol | 43.9 | 1568 | 1567 | 0.36±0.01 | 0.688±0.006 |
| Spathulenol | 44.27 | 1578 | 1577 | **9.715±0.007** | 0.84±0.03 |
| Globulol | 44.51 | 1584 | 1590 | - | 0.925±0.007 |
| Viridiflorol | 44.85 | 1593 | 1592 | **13.2±0.3** | **25.2±0.3** |
| Neryl 2-methylbutanoate | 45.07 | 1598 | n.r. | 0.96±0.08 | - |
| Ledol | 45.30 | 1604 | 1602 | **4.14±0.106** | **10.4±0.1** |
| Epicubenol | 46.2 | 1628 | 1627 | 0.305±0.007 | 0.629±0.009 |
| β-Eudesmol | 47.07 | 1651 | 1649 | - | 0.73±0.03 |
| Heptadecane | 48.65 | 1694 | 1700 | - | 0.22±0.008 |
| Naphtho[2,1-b]furan, dodecahydro-3a,6,6,9a-tetramethyl | 50.54 | 1767 | 1765* | 0.135±0.007 | 0.32±0.04 |
| Phenylethyl octanoate | 52.09 | 1848 | 1846 | 0.39±0.01 | - |
| 6-Oxoisoambrox | 52.22 | 1856 | 1855* | 0.79±0.02 | 0.754±0.001 |
| 8,13-epoxy- 15,16-Dinorlabd-12-ene | 52.77 | 1893 | - | 0.66±0.01 | - |
| Benzyl 3-phenylpropanoate | 53.30 | 1938 | - | 0.44±0.03 | - |
| geranyl-,alpha,-terpinene | 53.81 | 1984 | - | 0.71±0.04 | 0.24±0.02 |
| 2-Phenylethyl 3-phenylpropanoate | 54.41 | 2045 | - | **4.1±0.2** | - |
| geranyl 3-phenylpropanoate | 55.44 | 2170 | - | 0.64±0.04 | 1.16±0.01 |
| Tricosane | 56.28 | 2288 | 2300 | 0.42±0.04 | 0.68±0.03 |
| Heptacosane | 58.71 | 2683 | 2700 | - | 1.5±0.1 |
| Nonacosane | 60.13 | 2874 | 2900 | - | 1.7±0.09 |
| Total of identified compounds | | | | 58 ± 1 | 67.21 ± 0.06 |
| Monoterpene hydrocarbons | | | | 3.3 ± 0.2 | 0.526 ± 0.03 |
| Oxygen-containing monoterpenes | | | | 17.4 ± 0.3 | 17 ± 3 |
| Sesquiterpene hydrocarbons | | | | 3.1 ± 0.1 | 3 ± 1 |
| Oxygen-containing sesquiterpenes | | | | 30.1 ± 0.6 | 40.1 ± 0.8 |
| Diterpene hydrocarbons | | | | 0.76 ± 0.04 | 0.24 ± 0.02 |
| Others | | | | 4.27 ± 0.06 | 6.0 ± 0.3 |

[a] LRI, linear retention index determined on a SH- RXi-5ms fused silica column relative to a series of n-alkanes (C8–C40). [b] linear retention index reported in literature (Adams, 2017) or * retrived from NIST 2017.

## V.2 Extraction methods for obtaining cistus concretes

Besides the essential oil, other products can be obtained from *C. ladanifer*, namely the labdanum which is the resin exudated by the plant and can be obtained by different methods, most frequently by boiling with water and sodium carbonate; the absolutes corresponding to the extraction of the resin with organic solvents and concretes obtained by extracting the plant with organic solvents. Besides analyzing the essential oil of rock-rose in this work different concretes were prepared, namely from leaves and buds using hexane and ethanol. The results of the obtained yields are presented in Table 3. The highest yields for both leaves and buds were obtained using soxhlet extraction and ethanol as a solvent, namely 26.1% for leaves and 29.2% for the buds. For the leaves, extraction with hexane was higher using MW (11.4%). In general ultrasound was the extraction method that achieved the lowest extraction yields, with hexane performing the worst corresponding only to 5.62% and 7.39% for leaves and buds, respectively. In all tested extraction methods, ethanol allowed obtaining higher extraction yields as compared to hexane. With the exception of MW extraction with hexane, in general the buds resulted on higher yields compared to the leaves.

The obtained extracts were analyzed by GC-MS as well as submitted to biological activity evaluation, namely antioxidant and antimicrobial assays.

Table 3. Extraction yields for soxhlet, microwave and ultrasound.

| Extraction method | Solvent | Plant Part | Yield(%) | CV |
|---|---|---|---|---|
| **Soxhlet** | EtOH | leaves | 26.1 ± 0.8 | 3.4 |
| | | buds | 29 ± 1 | 4.1 |
| | Hexane | leaves | 9.7 ± 0.4 | 4.3 |
| | | buds | 11.3 ± 0.8 | 7.6 |
| **Microwave (MW)** | EtOH | leaves | 22.8 ± 0.9 | 4.2 |
| | | buds | 26.0 ± 0.3 | 1.2 |
| | Hexane | leaves | 11 ± 1 | 9.0 |
| | | buds | 8.6 ± 0.5 | 5.9 |
| **Ultrasound (US)** | EtOH | leaves | 18.8 ± 0.7 | 4.1 |
| | | buds | 21.5 ± 0.5 | 2.4 |
| | Hexane | leaves | 5.62 ± 0.01 | 1.0 |
| | | buds | 7.4 ± 0.3 | 4.5 |

## V.3. Chemical composition on concretes

The results obtained for the chemical composition of the concretes is shown in Tables 4 to 7. In general, it was verified that all extracts were much complex with more than one hundred peaks being integrated. As can be observed in Tables 4 to 7, all presented several peaks that were not identified as no match with a high percentage of confidence was retrieved from the NIST17. Table 4 shows the comparison of the results obtained using the three extraction methods for the buds extracted with hexane. Unfortunately, it was not possible to perform a triplicate of injection for the Soxhlet extract, even so it was possible to observe that this extract was the one presenting a higher number of identified compounds, as occurred also with the leaves (Table 5). In general, the identified compounds with higher content were long chain hydrocarbons. Interestingly, the MW extraction allowed obtaining a higher content of viridifloral as compared to the other methods. Figure 11 shows the chromatograms obtained using the 3 different extraction methods applied to buds extracted with hexane.

Table 4. Chemical composition determined by GC-MS of *Cistus ladanifer* buds extracted with hexane.

| Compounds | RT(min) | | | SOXHLET | MW | US |
|---|---|---|---|---|---|---|
| | | LRI calculated | LRI theoretical | | mean+ SD(%) | mean+ SD(%) |
| 6,6-Dimethylhepta-2,4-diene | 8.13 | 857 | - [a] | 0.128 | 0.065±0.008 | 0.066±0.002 |
| Cyclene | 10.28 | 921 | 926 | 0.184 | - | 0.036±0.002 |
| α-pinene | 10.71 | 933 | 932 | 1.478 | 0.21±0.05 | 0.33±0.04 |
| camphene | 11.25 | 948 | 946 | 0.946 | 0.028±0.007 | 0.159±0.023 |
| β-pinene | 12.30 | 976 | 974 | 0.026 | 0.028±0.01 | - |
| p-cymene + unknown | 14.09 | 1024 | 1020 | 0.102 | - | - |
| limonene | 14.24 | 1028 | 1024 | 0.047 | 0.38±0.09 | - |
| 2.6.6-Trimethylcyclohexanone | 14.44 | 1034 | - [a] | 1.676 | 1.2±0.1 | 1.193±0.008 |
| trans-Sabinene hydrate | 16.88 | 1098 | 1098 | 0.090 | - | - |
| Phenylethyl Alcohol | 17.45 | 1113 | 1107 | 0.195 | - | - |
| α-Campholenal + unkown | 17.91 | 1126 | 1122 | 0.540 | 0.22±0.04 | - |
| trans-Pinocarveol | 18.43 | 1140 | 1135 | 0.312 | 0.11±0.01 | 0.17±0.02 |
| trans-Verbenol | 18.63 | 1145 | 1140 | 1.133 | 0.5±0.1 | 0.48±0.02 |
| Pinocarvone | 19.31 | 1164 | 1160 | 0.154 | - | - |
| endo-Borneol | 19.43 | 1167 | 1165 | 0.549 | 0.21±0.02 | 0.2±0.03 |
| Terpinen-4-ol | 19.84 | 1178 | 1174 | 0.146 | 0.07±0.03 | - |
| ρ-Cymen-8-ol | 20.10 | 1185 | 1179 | 0.116 | - | - |
| (-)-Myrtenol | 20.54 | 1197 | 1194 | 0.263 | 0.15±0.01 | 0.105±0.006 |
| cis-Verbenone | 21.00 | 1210 | 1204 | 1.087 | 0.46±0.07 | 0.35±0.02 |
| cis-Carveol | 21.32 | 1219 | 1226 | 0.252 | 0.1±0.01 | - |
| Bornyl acetate | 23.70 | 1287 | 1284 | 1.509 | 0.7±0.1 | 0.44±0.01 |
| trans-Verbenyl acetate | 23.97 | 1295 | 1291 | 0.089 | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| 4-Hydroxy-3-methylacetophenone | 24.49 | 1310 | - [a] | 0.683 | - | - |
| Isopropyl 3-phenylpropanoate | 25.45 | 1339 | - [a] | 0.454 | - | - |
| Aromadendrene <allo-> | 29.52 | 1465 | 1464 | 0.072 | 0.11±0.01 | - |
| Ar-Curcumene | 30.17 | 1468 | 1479 | - | 0.07±0.03 | - |
| Viridiflorol | 33.59 | 1600 | 1592 | 2.782 | 4.4±0.2 | 3.14±0.07 |
| ledol | 33.93 | 1612 | 1602 | 1.055 | 1.4±0.4 | 1.03±0.02 |
| Hexadecanal | 39.75 | 1815 | 1811* | | | 0.14±0.004 |
| Neophytadiene | 40.38 | 1836 | 1837* | 0.127 | 0.214±0.002 | 0.18±0.01 |
| 6-Oxoisoambrox | 40.97 | 1852 | - [a] | 0.281 | 0.8±0.2 | 0.691±0.004 |
| 15.16-Dinorlab-12-ene. 8.13-epoxy- | 42.05 | 1894 | 1894* | 0.164 | 0.215±0.001 | 0.16±0.03 |
| Benzyl hydrocinnamic acid | 43.28 | 1941 | 1941 | 0.287 | - | - |
| Octadecanal | 45.66 | 2018 | 2021* | 0.619 | - | 1.07±0.05 |
| 2-Phenylethyl 3-phenylpropanoate | 46.28 | 2049 | 2049* | 3.218 | 2.5±0.4 | 1.4±0.1 |
| geranyl 3-phenylpropanoate | 49.05 | 2137 | - [a] | 0.349 | 1.14±0.08 | 0.94±0.06 |
| 3-Phenylpropyl 3-phenylpropanoate | 49.62 | 2157 | - [a] | 0.633 | 0.35±0.04 | 0.73±0.06 |
| Eicosanal | 51.43 | 2222 | - [a] | 0.913 | 0.43±0.06 | 1.4±0.3 |
| Heptacosane | 63.82 | 2695 | 2700 | 5.907 | 7±0.5 | 4.6±0.1 |
| Nonacosane | 69.20 | 2895 | - [a] | 6.040 | 8.6±0.8 | 6.9±0.3 |
| Total identified | | | | 34.61 | 33.2±0.4 | 29.8±0.3 |

[a] LRI, linear retention index determined on a SH- RXi-5ms fused silica column relative to a series of n-alkanes (C8–C40). [b] linear retention index reported in literature (Adams, 2017) or * retrived from NIST 2017.

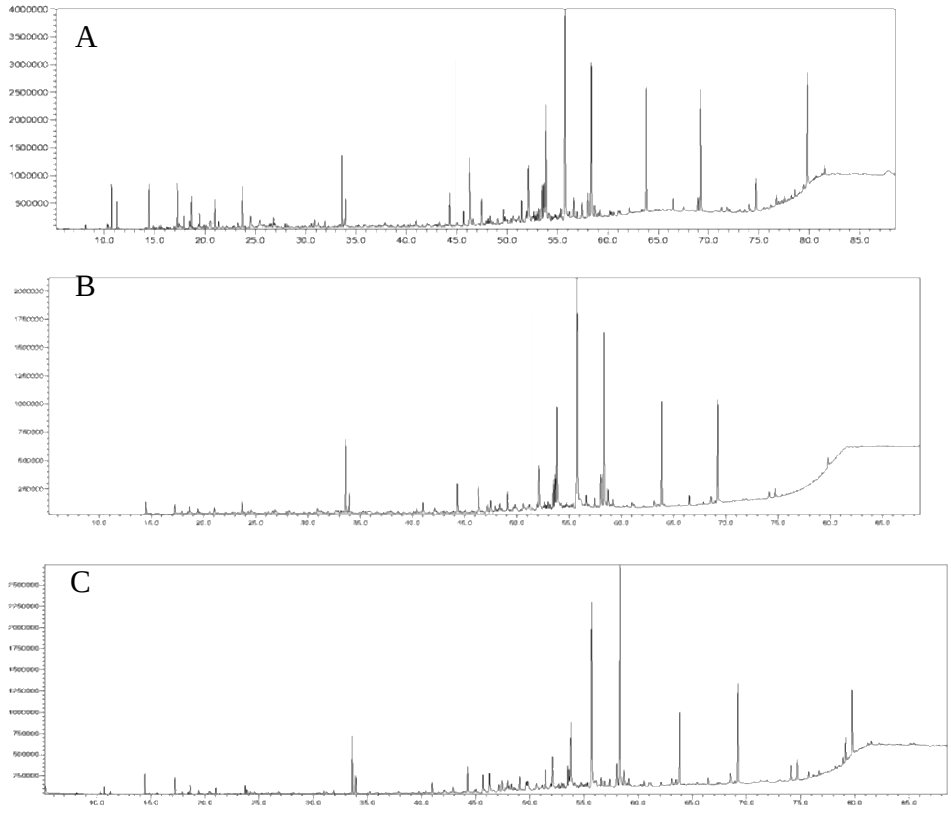


Figure 11. Chromatograms obtained by GC-MS of *Cistus ladanifer* buds extracted with hexane (A: soxhlet, B: MW, C: US).

Table 5. Chemical composition of *Cistus ladanifer* leaves extract with hexane determined by GC-MS.

| Compounds | RT(min) | | | SOXHLET | MW | US |
|---|---|---|---|---|---|---|
| | | LRI calculated | LRI theoretical | | mean+ SD(%) | mean+ SD(%) |
| 1,2,4,4-Tetramethyl-1-cyclopentene | 8.14 | 857 | 858* | 0.027 | - | 0.04±0.01 |
| α-pinene | 10.71 | 933 | 932 | 0.080 | 0.4±0.1 | 0.11±0.02 |
| camphene | 11.26 | 948 | 946 | 0.076 | 0.029±0.009 | 0.11±0.02 |
| Myrcene | 12.84 | 991 | 988 | - | 0.12±0.04 | |
| limonene | 14.28 | 1029 | 1024 | - | 1.1±0.4 | |
| 1.8-cineole | 14.39 | 1032 | 1026 | - | 0.23±0.05 | |
| 2.2.6-Trimethylcyclohexanone | 14.45 | 1034 | - [a] | 0.373 | 0.54±0.05 | 0.7±0.1 |
| α-Campholenal + unkown | 17.92 | 1126 | 1122 | 0.095 | - | 0.1±0.02 |
| trans-(-)-Pinocarveol | 18.43 | 1140 | 1135 | 0.232 | 0.12±0.05 | 0.16±0.05 |
| trans-Verbenol | 18.64 | 1146 | 1140 | 0.274 | 0.23±0.02 | 0.25±0.05 |
| Pinocarvone | 19.31 | 1164 | - [a] | 0.083 | - | 0.052±0.004 |
| endo-Borneol | 19.44 | 1167 | 1160 | 0.163 | 0.154±0.001 | 0.25±0.05 |
| cis-Pinocamphone | 19.74 | 1176 | 1172 | 0.045 | - | 0.04±0.01 |
| Myrtenol | 20.55 | 1197 | 1194 | 0.076 | - | 0.05±0.01 |
| cis-Verbenone | 21.01 | 1211 | 1204 | 0.240 | - | 0.15±0.02 |
| cis-Carveol | 21.34 | 1220 | 1226 | 0.070 | - | |
| Bornyl acetate | 23.70 | 1287 | 1284 | 0.315 | 0.22±0.05 | 0.29±0.06 |
| thymol | 23.88 | 1292 | 1289 | - | - | 0.35±0.06 |
| carvacrol | 24.22 | 1302 | 1298 | - | - | 0.7±0.2 |
| 4-Hydroxy-3-methylacetophenone | 24.51 | 1311 | 1323* | 0.275 | - | |
| Aromadendrene <allo-> | 29.59 | 1468 | 1464 | 0.074 | - | |
| Spathulenol | 33.13 | 1585 | 1577 | 0.070 | 0.11±0.07 | |
| Viridiflorol | 33.59 | 1600 | 1592 | 3.052 | 4±1 | 4±0.7 |
| Ledol | 33.93 | 1612 | 1602 | 1.302 | 1±0.3 | 1.2±0.2 |
| β-Eudesmol | 35.24 | 1658 | 1649 | 0.102 | 0.1±0 | 0.15±0.03 |
| Hexadecanal | 39.74 | 1805 | 1811 | 0.169 | 0.27±0.07 | 0.18±0.03 |
| Neophytadiene | 40.38 | 1829 | - [a] | 0.699 | 0.9±0.3 | 0.46±0.08 |
| 6-Oxoisoambrox | 40.97 | 1852 | - [a] | 0.636 | 0.51±0.05 | 0.9±0.1 |
| 15,16-Dinorlab-12-ene, 8,13-epoxy- | 42.04 | 1893 | 1894* | 0.102 | 0.27±0.01 | 0.15±0.02 |
| Octadecanal | 45.66 | 2018 | 2021* | 0.942 | 1.1±0.8 | 1.1±0.2 |
| 2-Phenylethyl 3-phenylpropanoate | 46.27 | 2039 | 2049* | 0.964 | 1.5±0.2 | 1.1±0.1 |
| 2-Nonadecanone | 48.12 | 2104 | 2100* | - | 0.4±0.1 | |
| geranyl 3-phenylpropanoate | 49.05 | 2137 | - [a] | - | 0.8±0.1 | 0.6±0.04 |
| Eicosanal | 51.43 | 2222 | 2224* | 1.622 | 2±1 | 1.7±0.5 |
| tricosane | 53.45 | 2299 | 2300 | 0.295 | 0.22±0.05 | |
| pentacosane | 58.70 | 2495 | 2500 | 0.207 | 0.3±0.1 | |
| Heptacosane | 63.83 | 2695 | 2700 | 8.545 | - | 6.92±0.06 |
| Nonacosane | 69.27 | 2897 | 2900 | 24.680 | 21.1±0.5 | 22.6±0.6 |
| | | | | | | |
| Total identified | | | | 45.38 | 37±5 | 44.4±0.6 |

[a] LRI, linear retention index determined on a SH- RXi-5ms fused silica column relative to a series of n-alkanes (C8–C40). [b] linear retention index reported in literature (Adams, 2017) or * retrived from NIST 2017.

The chromatograms of the hexane concretes obtained using the 3 methods applied to the leaves are shown in Figure 12. As can be observed in Table 5, similarly to the buds also a higher content of viridiflorol was obtained using the MW extraction. As well, a high number of compounds were not identified, corresponding to around 70% of the extracts. Both in the leaves and buds, three peaks with retention times approximately of 53.8, 55.7 and 58.3 minutes accounted for 35% of the extracts. The mass spectra of these three compounds are shown in Figure 13. As it can be observed, peaks at 55.7 min and 58.3 min showed spectra with similar fragments and possibly could be of the same structural family.

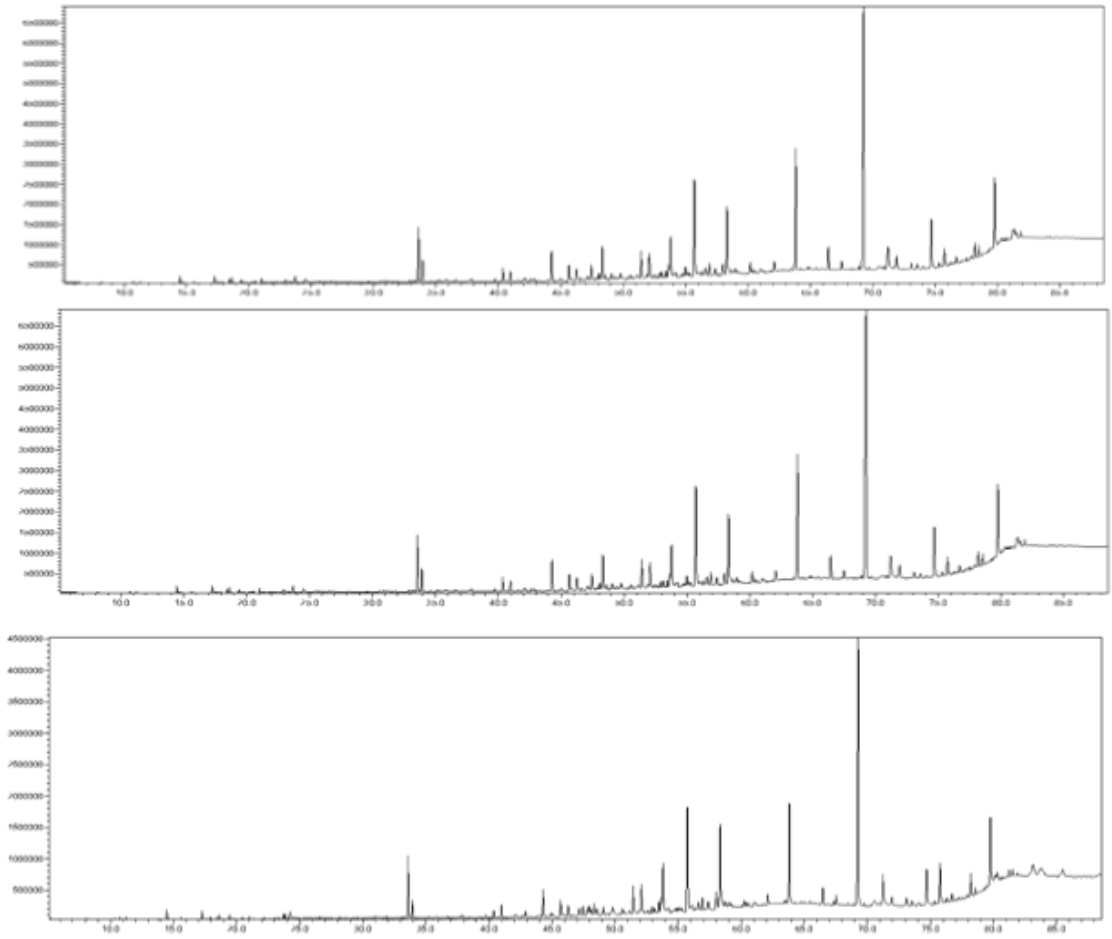

Figure 12. Chromatograms obtained by GC-MS of *Cistus ladanifer* buds extracted with hexane (up: soxhlet, midle: MW, bottom: US).

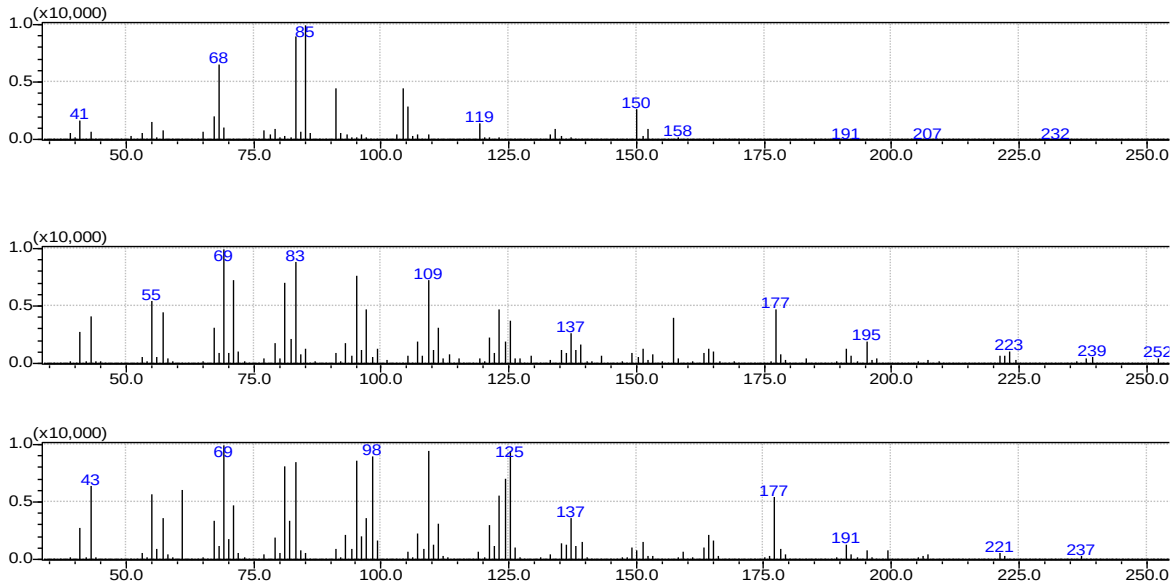


Figure 13. Mass spectra obtained for non-identified compounds with retention time of 53.8, 55.7 and 58.3 minutes (up, middle and bottom figures, respectively).

Table 6 and 7 shows the results of the GC-MS analyses of the ethanolic extracts of buds and leaves, respectively. As can be observed, for the buds MW and US extraction allowed for a higher percentage of identified compounds to be extracted. In particular, MW extraction resulted on a higher content of viridiflorol in the extracts. In the leaves, curiously MW was the extraction method that allowed for a higher number of compounds to be identified, although those corresponded to a lower percentage of total identified compounds. This can be related to the fact that some compounds are present in very low amounts, such as trans-verbenol, borneol and cis-verbenone all around 0.2%, thus possibly being detected in the most efficient extraction method.

Table 6. Chemical composition of *Cistus ladanifer's* buds ethanolic extract determined by GC-MS.

| Compounds | RT(min) | LRI calculated | LRI theoretical | SOXHLET (%) | MW (%) | US (%) |
|---|---|---|---|---|---|---|
| 2,6,6-Trimethylcyclohexanone | 12.93 | 1037 | - | 1.69 | 1.30 | 1.02 |
| trans-Verbenol | 16.33 | 1149 | 1140 | 0.64 | 0.33 | - |
| borneol | 16.99 | 1171 | 1165 | 0.27 | 0.22 | - |
| Verbenyl ethyl ether | 17.42 | 1185 | 1186* | 0.39 | - | 0.08 |
| myrtenol | 17.88 | 1201 | 1194 | 0.21 | - | - |
| cis-Verbenone | 18.27 | 1214 | 1204 | 0.88 | 0.27 | 0.14 |
| Bornyl acetate | 20.42 | 1290 | 1284 | 0.70 | 0.65 | 0.14 |
| Viridiflorol | 28.43 | 1606 | 1592 | 1.51 | 4.56 | 3.83 |
| Ledol | 28.72 | 1618 | 1602 | 0.28 | 1.17 | 1.29 |
| Neophytadiene | 34.30 | 1838 | - [a] | 0.24 | 0.48 | 0.43 |
| 6-Oxoisoambrox | 34.96 | 1863 | - [a] | 0.38 | 0.75 | 1.11 |
| 15,16-Dinorlab-12-ene, 8,13-epoxy- | 35.93 | 1899 | 1894* | 0.36 | - | 0.60 |
| 2-Phenylethyl 3-phenylpropanoate | 39.87 | 2044 | 2049* | 0.60 | 2.11 | 2.17 |
| octadecanol | 40.95 | 2083 | 2084* | 0.31 | 0.68 | 0.77 |
| geranyl 3-phenylpropanoate | 42.53 | 2140 | - [a] | 0.57 | 1.32 | 1.27 |
| tricosane | 46.79 | 2298 | 2300 | 2.15 | 2.31 | 2.27 |
| Heptacosane | 56.86 | 2699 | 2700 | 4.43 | 5.04 | 6.61 |
| Nonacosane | 61.44 | 2898 | 2900 | 2.48 | 2.85 | 6.79 |
| | | | | | | |
| Total identified | | | | 18.08 | 24.02 | 28.54 |

Table 7. Chemical composition of *Cistus ladanifer's* leaves ethanolic extract determined by GC-MS.

| Compounds | RT(min) | LRI calculated | LRI theoretical | SOXHLET (%) | MW (%) | US (%) |
|---|---|---|---|---|---|---|
| 2,6,6-Trimethylcyclohexanone | 12.96 | 1037 | - [a] | - | 0.86 | - |
| trans-Verbenol | 16.37 | 1149 | 1140 | - | 0.22 | - |
| borneol | 17.02 | 1171 | 1165 | - | 0.20 | - |
| cis-Verbenone | 18.31 | 1214 | 1204 | - | 0.21 | - |
| Bornyl acetate | 20.42 | 1290 | 1284 | 0.47 | 0.53 | 0.36 |
| Carvacrol | 20.842 | 1306 | 1298 | - | - | 0.49 |
| 4-(p-Hydroxyphenyl)-2-butanol | 27.57 | 1570 | - [a] | 2.93 | 9.16 | 10.12 |
| Viridiflorol | 28.431 | 1606 | 1592 | 7.98 | 6.22 | 5.07 |
| Ledol | 28.715 | 1618 | 1602 | 3.84 | 2.18 | 1.49 |
| Neophytadiene | 34.30 | 1838 | - [a] | 1.35 | 1.11 | 0.71 |
| 6-Oxoisoambrox | 34.96 | 1863 | - [a] | 1.31 | 1.22 | 1.56 |
| 15,16-Dinorlab-12-ene, 8,13-epoxy- | 35.93 | 1899 | 1894* | 0.46 | 0.54 | - |
| 2-Phenylethyl 3-phenylpropanoate | 39.90 | 2044 | 2049* | - | 2.47 | 1.84 |

| | | | | | | |
|---|---|---|---|---|---|---|
| geranyl 3-phenylpropanoate | 42.525 | 2140 | - [a] | 0.34 | 1.82 | 1.03 |
| tricosane | 46.791 | 2298 | 2300 | 0.87 | 2.07 | 0.68 |
| Heptacosane | 56.856 | 2699 | 2700 | 7.53 | 2.24 | 6.26 |
| Nonacosane | 61.436 | 2898 | 2900 | 9.07 | 0.50 | 12.44 |
| | | | | | | |
| Total identified | | | | 33.22 | 31.55 | 42.04 |

[a] LRI, linear retention index determined on a SH- RXi-5ms fused silica column relative to a series of n-alkanes (C8–C40). [b] linear retention index reported in literature (Adams, 2017) or * retrived from NIST 2017.

In general, despite slightly higher extraction yields being obtained with Soxhlet extraction, MW allowed obtaining similar results and in some cases even a higher amount of some compounds associated with aroma profile in the extracts. Moreover, it should be considered that soxhlet approach uses a very long period of extraction (6 hours), thus being associated with a high energy cost. Therefore, for MW was selected for further optimization studies.

### V.4. Antioxidant activity

To determine the antioxidant potential, two different methods were applied to analyze the twelve samples, namely the 2,2-diphenyl-1- picrylhydrazyl (DPPH) and the reduction power assays, both expressed in terms of $EC_{50}$ value (concentration able to exert 50% of antioxidant activity), in mg/mL. In table 9 it is possible to observe the obtained results for each sample according to the extraction technique. Most of the extracts were able to exert antioxidant effects in the DPPH method. Nevertheless, the highest radical scavenging activity was displayed by *C. ladanifer* leaves ethanolic extract using the microwave assisted extraction with a lower $EC_{50}$ value of 0.152 ± 0.005 mg/mL, being the extracts obtained from the Soxhlet using hexane as the extracting solvent. In general, the ethanolic extracts exhibited the strongest potential, a fact attributed to the compounds extracted with this solvent, mainly phenolics that present higher affinity to polar solvents.

Considering the reducing power assay, all the extracts presented reducing capacity, standing out the samples obtained from the ultrasound assisted extraction with ethanol for the buds samples. Regarding the three different techniques, in general, for the DPPH assay, the microwave extraction was the most effective one, followed by the ultrasound and the Soxhlet. On the other hand, for the reducing power, the Soxhlet was, in general the most efficient technique, followed by the microwave and the ultrasound assisted extractions. These

differences can be attributed to the different mechanisms of action of the two applied methods, that is through the transfer of an hydrogen atom, while in the reducing power the antioxidant activity is achieved through electrons transfer.

Table 9. Antioxidant activity of the different samples (mg/mL).

| EXTRACTION METHOD | SOLVENT | PLANT PART | DPPH $EC_{50}$ | REDUCING POWER $EC_{50}$ |
|---|---|---|---|---|
| **SOXHLET** | EtOH | leaves | 1.4±0.4 | 0.66±0.08 |
| | | buds | 3.7±0.3 | 0.9±0.3 |
| | Hexane | leaves | 6.0±0.5 | 0.8±0.1 |
| | | buds | - | 0.94±0.07 |
| **MICROWAVE** | EtOH | leaves | 0.152±0.005 | 0.68±0.03 |
| | | buds | 0.24±0.06 | 0.31±0.01 |
| | Hexane | leaves | - | 1.01±0.03 |
| | | buds | - | 0.90±0.02 |
| **ULTRASOUND** | EtOH | leaves | 0.36±0.1 | 0.23±0.02 |
| | | buds | 0.17±0.02 | 0.15±0.01 |
| | Hexane | leaves | 3.4±0.4 | 4.0±0.9 |
| | | buds | - | 8.2±1.9 |

Positive control: Trolox (DPPH and Reducing power $EC_{50}$ values: 0.03±0.01and 0.041±0.01 mg/mL).

## V.5. Antibacterial activity

Table 10 displays the results of the obtained extracts against several pathogenic bacterial strains. All the tested extracts revealed inhibition activity against these Gram-positive and Gram-negative bacteria.

The ethanolic extracts using the microwave assisted extraction revealed stronger capacity has on the growth of *E. Cloacae, E. coli* and MRSA with MIC values of 2.5, 5 and 1.25 mg/mL, respectively. For *S. enterocolitica,* the most interesting activity was obtained with ethanolic extracts from the buds using microwave. On the other hand, extracts from the buds and leaves obtained with hexane as a solvent, leaves ethanolic extract using ultrasound and leaves extract using the soxhlet techniques and hexane as a solvent showed the same activity against *Y. enterocolitica* with a MIC value of 0.6 mg/mL.

For the Gram positive bacteria, for both *B. cereus* and *L. monocytogenes*, the highest antibacterial activity (MIC = 0.3 mg/mL) was achieved with the ethanolic extracts from the leaves extracted with soxhlet and from the buds extracted with microwave and hexane as

solvent. For *S. aureus*, all the extracts using microwave and the ethanolic extract from the leaves using soxhlet showed the same interesting activity (MIC = 1.25 mg/mL).

Regarding *E. coli*, using microwave as a method of extraction, the best activity was obtained with extracts from buds with ethanol, while using ultrasound, the best potential was achieved with leaves with ethanol, the same as for *P. mirabilis and p. aeruginosa* with MICs equal to 1.25mg/mL and 5mg/mL, respectiveley. When using the soxhlet as method of extraction, the sample that presented the best activity was the buds extract with hexane for *E. coli* and the buds extract with ethanol for *P. mirabilis P. aeruginosa*. The most interesting inhibition activities against *P. aeruginosa and E. faecalis* was obtained with ethanolic extracts from the buds of *C.ladanifer* extracted with soxhlet. All the extracts using soxhlet and microwave showed the same activity against *L. monocytogenes* (MIC=5 mg/mL), except the extracts using ultrasound, where only leaves with ethanol presented the same inhibition potential.

Table 10. Antimicrobial Activity of the *C. ladanifer* samples (mg/mL).

| ***Extraction method*** | ***Soxhlet*** | | | | ***Microwave*** | | | | ***US*** | | | | *Positive controls* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Solvent* | EtOH | | Hexane | | EtOH | | Hexane | | EtOH | | Hexane | | Streptomycin 1 mg/mL | Methicilin 1 mg/mL | Ampicillin 20 mg/mL |
| *Plant part* | Leaves | buds | leaves | buds | leaves | buds | leaves | buds | leaves | Buds | leaves | buds | | | |
| Foodborne bacteria | | | | | | | | | | | | | | | |
| *E. Cloacae* | 10/>10 | 10/>10 | >10 | >10 | 2.5/>10 | 10/>10 | >10 | 10/>10 | 10/>10 | >10 | >10 | >10 | 0.007/0.007 | n.t. | 0.15/0.15 |
| *E. coli* | 10/>10 | 10/>10 | 10/>10 | 10/>10 | 5/>10 | 5/>10 | 10/>10 | 10/>10 | 10/>10 | 10/>10 | >10 | >10 | 0.01/0.01 | n.t. | 0.15/0.15 |
| *P. aeruginosa* | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | 0.06/0.06 | n.t. | 0.63/0.63 |
| *S. enterocolitica* | 2.5/>10 | 2.5/>10 | 2.5/>10 | 2.5/>10 | 2.5/>10 | 1,25/>10 | 2.5/>10 | 2.5/>10 | 2.5/>10 | 10/>10 | >10 | 10/>10 | 0.007/0.007 | n.t. | 0.15/0.15 |
| *Y. enterocolitica* | 5/>10 | 5/>10 | 0.6/>10 | 5/>10 | 10/>10 | 2.5/>10 | 2.5/>10 | 2.5/>10 | 0.6/>10 | >10 | 0.6/>10 | 0.6/>10 | 0.007/0.007 | n.t. | 0.15/0.15 |
| *B. cereus* | 0.3/>10 | 0.3/>10 | 1.25/>10 | 2.5/>10 | 1.25/>10 | 1.25/>10 | 0.6/>10 | 0.3/>10 | 2.5/>10 | 2.5/>10 | 1.25/>10 | 0,6/>10 | 0.007/0.007 | n.t. | n.t. |
| *L. monocytogenes* | 0.3/>10 | 0.3/10 | 0.6/>10 | 1.25/10 | 0.6/>10 | 1.25/10 | 1.25/10 | 0.3/10 | 1.25/>10 | >10 | >10 | >10 | 0.007/0.007 | n.t. | 0.15/0.15 |
| *S. aureus* | 1.25/>10 | 1.25/10 | 2.5/>10 | 5/>10 | 1.25/10 | 1.25/10 | 1.25/10 | 1.25/>10 | 2.5/>10 | 2.5/>10 | >10 | 10/>10 | 0.007/0.007 | 0.007/0.007 | 0.15/0.15 |
| Clinical bacteria | | | | | | | | | | | | | | | |
| *E. coli* | 1.25/>10 | 2.5/>10 | 2.5/>10 | 1.25/>10 | 2.5/>10 | 1.25/>10 | 2.5/>10 | 2.5/>10 | 1.25/>10 | >10 | >10 | >10 | <0.15/<0.15 | <0.0078/<0.0078 | n.t |
| *K. pneumoniae* | 10/>10 | 10/>10 | >10 | >10 | 10/>10 | 10/>10 | >10 | 10/>10 | 10/>10 | >10 | >10 | >10 | 10/>10 | <0.0078/<0.0078 | n.t |
| *M. morganii* | 2.5/>10 | 5/>10 | 10/>10 | 10/>10 | 5/>10 | 5/>10 | 10/>10 | 10/>10 | 2.5/>10 | >10 | >10 | >10 | >10/>10 | <0.0078/<0.0078 | n.t |
| *P. mirabilis* | 5/>10 | 5/>10 | 10/>10 | 10/>10 | 5/>10 | 5/>10 | 10/>10 | 10/>10 | 5/>10 | >10 | >10 | >10 | <0.15/<0.15 | <0.0078/<0.0078 | n.t |
| *P. aeruginosa* | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | 10/>10 | >10 | >10 | >10/>10 | <0.0078/<0.0078 | n.t |
| *E. faecalis* | 1.25/>10 | 0.6/>10 | 1.25/>10 | 5.0/10 | 10/>10 | 1.25/>10 | 1.25/>10 | 2.5/>10 | 1.25/>10 | 10/>10 | >10 | >10 | <0.15/<0.15 | n.t | <0.0078/<0.0078 |
| *L. monocytogenes* | 5/>10 | 5.0/10 | 5.0/10 | 5.0/10 | 5.0/10 | 5.0/10 | 5.0/10 | 5.0/10 | 5.0/10 | >10 | >10 | >10 | <0.15/<0.15 | <0.0078/<0.0078 | n.t |
| *MRSA* | 2.5/>10 | 5/>10 | 10/>10 | 2.5/>10 | 1.25/10 | 2.5/10 | 2.5/>10 | 10/>10 | 2.5/>10 | 10/>10 | >10 | >10 | <0.15/<0.15 | n.t | 0.25//0.5 |

MRSA – methicilin resistant *S. aureus* ; n.t. – not tested.

In fact, the presence of mono and sesquiterpenes, which are the most abundant components in the samples, have previously been described as strong antimicrobial agents.

According to the study of (Morales-Soto et al., 2015), due to the presence of limonene and bornyl acetate, a relevant effect was observed against *E. coli* and *P. aeruginosa,* while *αThujene* and *caryophyllene* showed an effect against *S. enterocolitica* and *E. cloacae.* These authors also related that Pinocarvone was the compound who was responsible of the effect against *K. pneumoniae* and *B. cereus.*

In a study performed by (Tavares, Martins, Miguel, et al., 2020), *C. ladanifer* showed antibacterial activity against *E. coli* and *S. aureus.* According to (Barrajón-Catalán et al., 2010), *C. ladanifer* leaves aqueous extracts showed antibacterial activity against *S. aureus* and *E. coli* with MICs values of 0.154 and 0.900 mg/mL, respectively.

In another work, ((Benayad et al., 2013), showed that *C. ladanifer* hexane extracts inhibited some bacterial strains such as *Y. enterocolitica, E. coli* and *S. aureus*, and no activity was recorded against *P. aeruginosa and K. pneumoniae*.

The ethanolic extract from *C. ladanifer* exhibited also an important antibacterial effect against *S. aureus* MIC=1.56mg/mL in the work of (Benali et al., 2020) while for *P. mirabilis* no effect was obtained.

### V.6. Antifungal activity

In Table 10, the results from the twelve extracts against two different fungi (*Aspergillus brasiliensis and Aspergillus fumigatus*) can be visualized.

From the obtained MIC and MFC, hexane extracts as extracting solvent and the soxhlet extraction technique for both leaves and buds showed the most interesting inhibition activities against *A. brasiliensis* (MIC = 0.625 mg/mL) while the ethanolic extracts from the buds using microwave assisted extraction presented the weakest potential, exhibiting the highest MIC value of 5.0 mg/mL.

Regarding *A. fumigatus*, ethanolic extracts from the buds of *C. ladanifer* extracted with soxhlet presented the lowest MIC value ( MIC = 1.25 mg/mL) and consequently the strongest inhibition capacity, while the extracts using ultrasound and hexane as a solvent were not as efficient as the other extracts (MIC = 10 mg/mL).

Concerning the microwave assisted extraction; all the extracts presented the same inhibition activity against *A. brasiliensis* (MIC = 5mg/mL) while for *A. fumigatus*, with the ethanolic extract from the buds presenting the lowest activity when compared to the other extracts.
When applying the ultrasound assisted extraction, also, all the extracts presented the same activity against both the fungi (MIC = 10 mg/mL), except the ethanolic extracts from the leaves which presented the most interesting inhibition activity (MIC=2.5 mg/mL). On the other hand, the samples extracted by soxhlet and using hexane as the extractin solvent, extarcts from both leaves and buds showed the same inhibition potential, but, also the strongest one (MIC = 0.625 mg/mL) against *Aspergillus brasiliensis.* For *A. fumigatus*, it was the ethanolic extract from the buds who revealed the lowest MIC value (1.25 mg/mL).
Although having only found one similar studies in the literature regarding the antifungal activity of these parts of *C. ladanifer*, the ones obtained in this study revealed highest potential against fungi than the ones obtained by (Bouamama et al., 2006) for *A. fumigatus.*

Table 10. Antifungal activity of the different extracts of *C. ladanifer* (mg/mL).

| **EXTRACTION METHOD** | **Solvent** | **Plant part** | ***Aspergillus brasiliensis*** | ***Aspergillus fumigatus*** |
|---|---|---|---|---|
| **SOXHLET** | EtOH | leaves | - | - |
| | | buds | 1.25/5 | 1.25/2.5 |
| | Hexane | leaves | 0.625/5 | 2.5/5 |
| | | buds | 0.625/2.5 | 2.5/5 |
| **MICROWAVE** | EtOH | leaves | 5.0/10 | 2.5/5 |
| | | buds | 5.0/10 | 5.0/10 |
| | Hexane | leaves | 5.0/10 | 2.5/10 |
| | | buds | 5.0/10 | 2.5/5 |
| **ULTRASOUND** | EtOH | leaves | 2.5/5 | 2.5/10 |
| | | buds | 10/>10 | 10/>10 |
| | Hexane | leaves | 10/>10 | 10/>10 |
| | | buds | 10/>10 | 10/>10 |

## V.7. Optimization

Microwave assisted extraction (MAE) has been considered one of the most promising methods to obtain plant extracts. For this reason, different extraction parameters such as potency, time and temperature were varied to understand the relationships between the independent parameters and their influence on the extraction process from *Cistus ladanifer*

leaves and buds. For this, an experimental design was implemented, consisting of a17 run Bex-Behnken model with 5 repetitions of the center point to improve the precision.

### V.7.1. Bud´s optimization

The Table 11 shows the experimental design in random order for the buds, including the three varying factors, namely temperature ($\chi_1$ (A) varying between 0 and 60 ºC), time ($\chi_2$ (B) varying between 2 and 30 minutes), and microwave power ($\chi_3$ (C) varying between 50 and 800 W), as well as the responses, namely dry yield ($R_1$), DPPH antioxidant activity ($R_2$) and RP antioxidant activity ($R_3$). The green highlighted factors correspond to the center point repetitions.

Table 11. Experimental design of the MAE extraction of buds.

| Run | Temperature (ºC) $\chi_1$ | Time (min) $\chi_2$ | Power (W) $\chi_3$ | Dry Yield (g/100g) $R_1$ | DPPH $EC_{50}$ $R_2$ | RP $EC_{50}$ $R_3$ |
|---|---|---|---|---|---|---|
| 1 | 60 | 2 | 425 | 20.623 | 1.07 | 0.43 |
| 2 | 50 | 2 | 50 | 17.111 | 0.062 | ~~0.24~~ |
| 3 | 40 | 30 | 425 | 19.293 | 0.8 | 0.334 |
| 4 | 50 | 30 | 50 | 23.039 | 0.39 | 0.31 |
| 5 | 50 | 16 | 425 | 21.759 | ~~1.26~~ | 0.58 |
| 6 | 50 | 2 | 800 | 19.815 | 0.3 | 0.32 |
| 7 | 40 | 16 | 50 | 21.0368 | 0.38 | 0.36 |
| 8 | 50 | 16 | 425 | 21.965 | 0.5 | 0.35 |
| 9 | 50 | 16 | 425 | ~~16.056~~ | 0.78 | 0.39 |
| 10 | 40 | 2 | 425 | 17.736 | 1.3 | 0.59 |
| 11 | 60 | 16 | 800 | 21.530 | 1.6 | 0.192 |
| 12 | 40 | 30 | 800 | 21.623 | 0.54 | 0.29 |
| 13 | 50 | 16 | 425 | 20.461 | 0.66 | 0.235 |
| 14 | 40 | 16 | 800 | 19.520 | 0.5 | 0.51 |
| 15 | 60 | 16 | 50 | ~~25.634~~ | 0.8 | 0.44 |
| 16 | 60 | 30 | 425 | ~~27.315~~ | ~~0.16~~ | 0.12 |
| 17 | 50 | 16 | 425 | 22.342 | 0.77 | 0.425 |

Considering the first response, ($R_1$), the dry yield, three outliers were detected and thus their values ignored, namely run 9, run 15 and run 16. This was done to improve the fitting and outcome of the model. Still, after ignoring these outliers, no transformation was needed for the model, which was considered significant, while the lack of fit was not significant. Overall, the $R^2$ did not go over 0.57 while the adjusted $R^2$ scored 0.44, using a linear model.

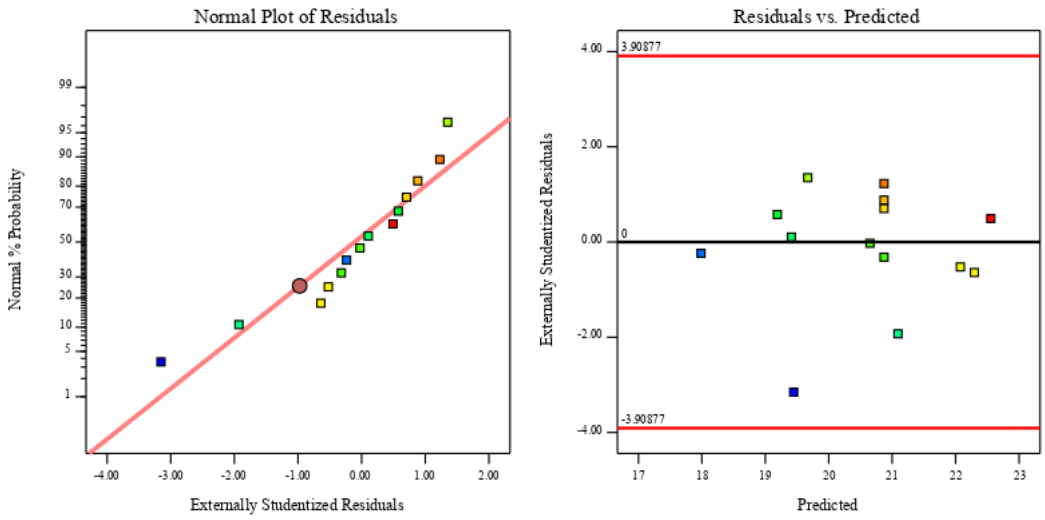


Figure 14. Plots of the residuals and residual vs. predicted.

Figure 14 shows the plots of the residuals after deleting the outliers, showing a homogenous distribution, albeit being skewed, while the residuals vs. predicted plots are within the inferior and superior limit, with only one residual being near the inferior limit (run 2). The coded equation for the model was $Yield = 20.87 + 0.92 * \chi_1 + 1.55 * \chi_2 - 0.1290 * \chi_3$. This shows that $\chi_2$ (time) was the factor with the highest impact in the yield of the extraction, which was kind of expected due to longer extraction periods allowing for a higher obtention of compounds.

Figure 16 shows the optimal points obtained through the model, in which temperature is set at the highest, around 60 ºC, time at 27 minutes and a low power of 333 W, showing that extraction with microwave technology does not need high intensities, rather a long time and high temperature. At these points, the yield is expected to reach 23 g/100 g of extract yield.

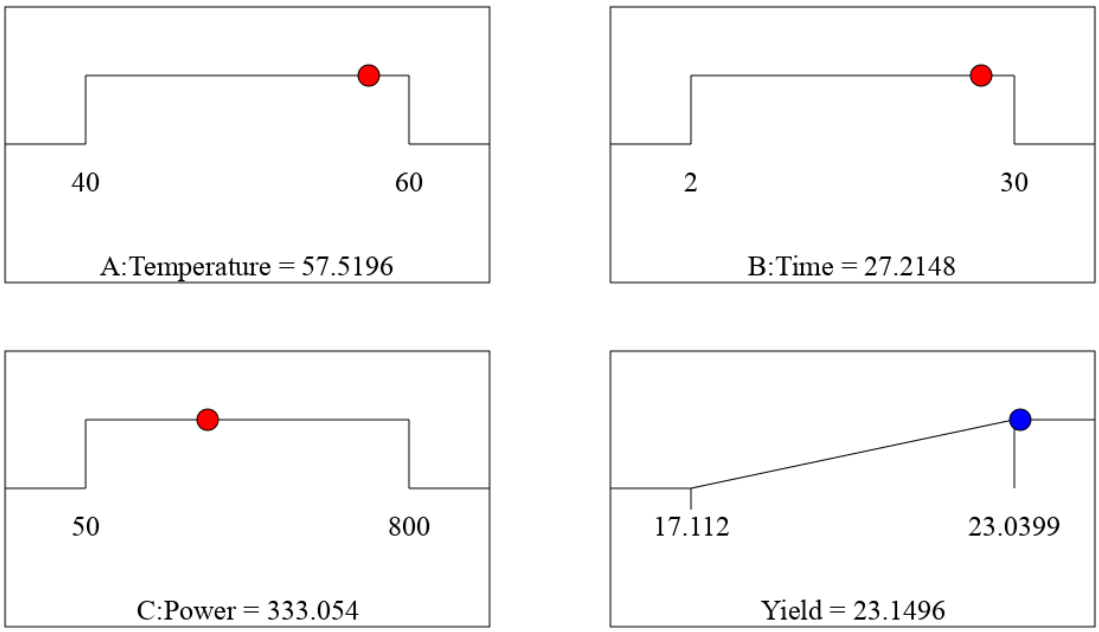


Figure 15. Optimal points and yield prediction at these points.

Due to the model being linear, interactions between two factors could not be plotted, thus, in figure 16, each factor is plotted individually. Temperature $\chi_1$, shows a constant increase in yield over the 60 minutes, being the second most important factor in terms of yield, as confirmed by the coded equation. Time, $\chi_2$, which was the second most important equation is also shown to increase the yield while power, which was the least important factor shows an almost constant extractability yield from 50 to 800 W. Considering all these effects, microwave power is not a determinant factor for the optimization of yield amounts and could be fixed with variation in other factors like soli to liquid ratio, among others.

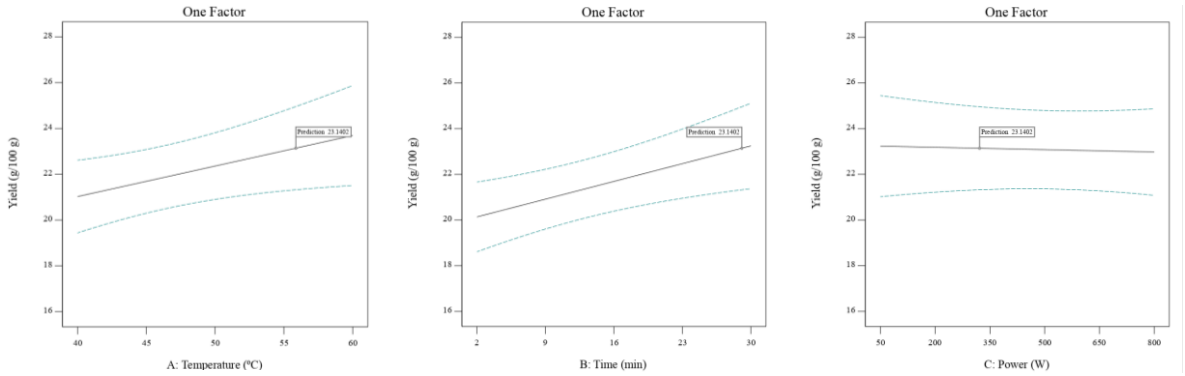


Figure16. Graphs of the individual factors of the dry yield of the buds.

Considering the DPPH activity of each of the runs, a quadratic model was fitted, showing non-significant lack of fit and a coded equation of $DPPH = 0.6775 + 0.3543 * \chi_1 + 0.1677 * \chi_2 + 0.1635 * \chi_3 + 0.4435 * \chi_1\chi_2 + 0.17 * \chi_1\chi_3 - 0.0220 * \chi_2\chi_3 + 0.5990 * \chi_1^2 + 0.1020 * \chi_2^2 - 0.4565 * \chi_3^2$, in which the most relevant factor is the temperature, followed by both other factors with similar relevance. The $R^2$ was 0.9562 and the adjusted $R^2$ was set at 0.8773. Considering the plots of residuals (Figure 17), these seem to be normal due to being quite close to the trendline, while none of the runs are above the upper and lower threshold.

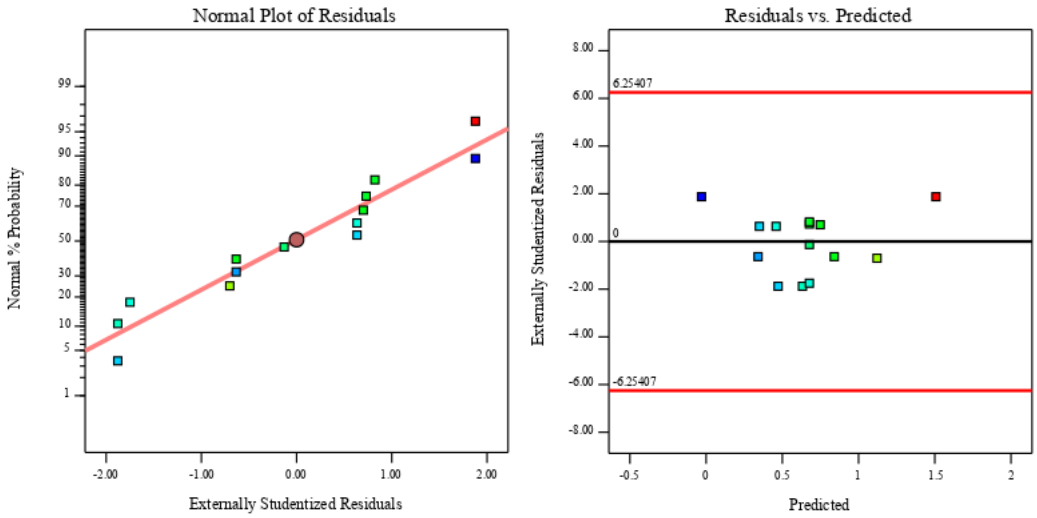


Figure 17. Plots of the residuals and residual vs. predicted.

Due to the DPPH being expressed in terms of $EC_{50}$ (concentration that quenches 50% of the radicals), the lowest concentrations in the response show higher antioxidant activity, the minimize function was used. Thus, the optimal values, that show lower $EC_{50}$ values were 48 ºC, 17 minutes and 50 W of microwave power (Figure 19). Thus, low intensities of extraction power increase the antioxidant activity while central values of temperature and power are the best, also contributing to this.

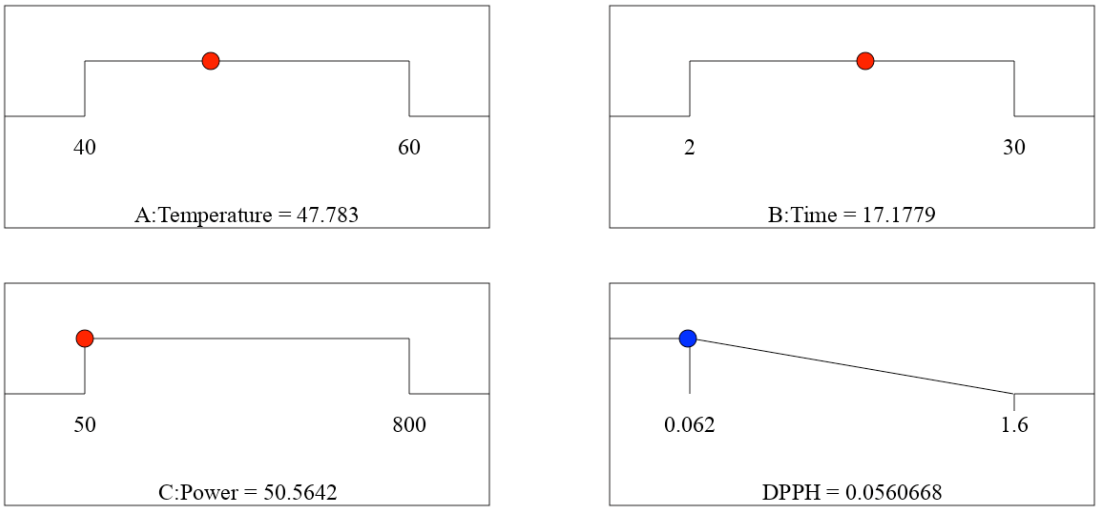


Figure 18. Optimal points and yield prediction of the optimal points for the DPPH of the buds.

The quadratic model obtained allowed the representation of the response to be plotted in a surface, which are shown in Figure 19. The first graph shows a blue valley in the central values of time and temperature, showing that, as seen in Figure 15, central values of these two factors favor the lowest $EC_{50}$ values. Similarly, in the central graph, the central values also show a low region, on the axis of the temperature, while for the power, central values show higher $EC_{50}$ values, and thus, the region with the lowest values are shown near the 50 W. The graph on the right, which plots power and time shows that the interaction between these two factors does not influence much the outcome, with lower powers favoring low DPPH values, while higher intensities reduce this effect.

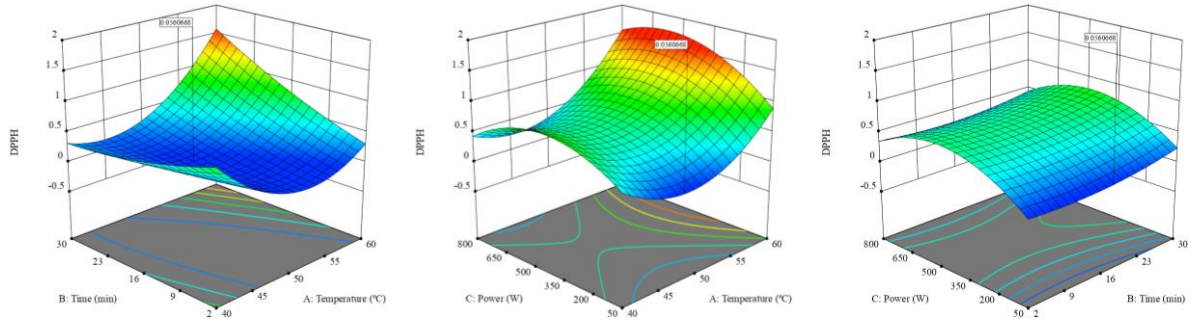


Figure 19. Plots of the individual factors for the DPPH assay of the buds.

Overall, DPPH values were favored by low intensities of microwaves and central values of temperature and time.

For the RP assay, run 2 was deleted due to being an outlier, and the obtained model was a linear one, classified as significant with a non-significant lack of fit, but the $R^2$ and the adjusted $R^2$ did now go over 0.474 and 0.342, respectively. The coded equation was $RP = 0.3760 - 0.0765 * \chi_1 - 0.1002 * \chi_2 - 0.0392 * \chi_3$, showing that time was the most important factor, followed by temperature, although with low impact, showing that for RP, the three chosen factors did not highly influence the antioxidant activity.

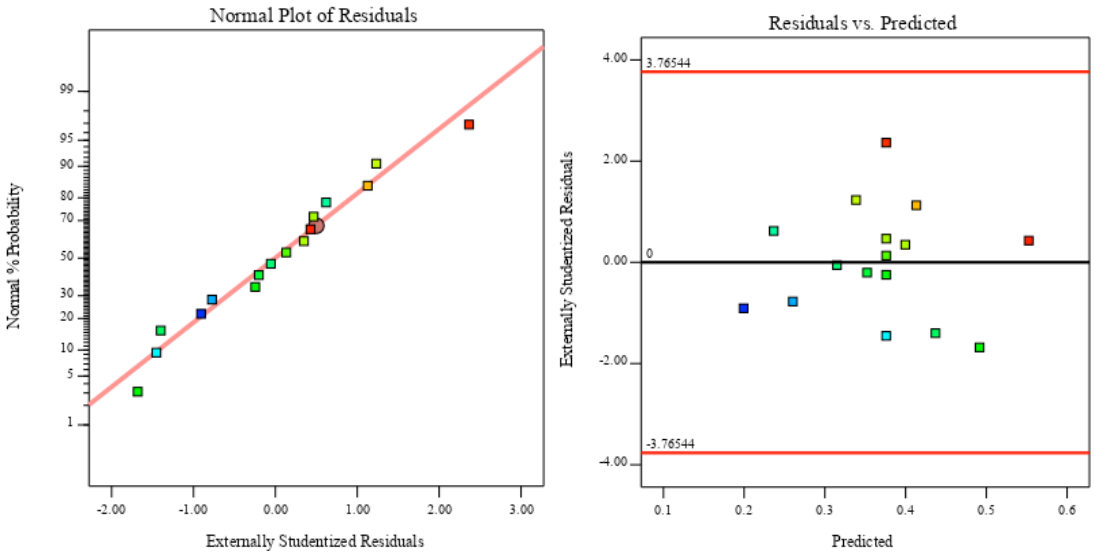


Figure 20. Plots of the residuals and residual vs. predicted.

In figure 20, the plot of residuals shows that the runs are distributed over the trendline, while the residuals vs. predicted values are within the upper and lower bounds. The minimize function was used to calculate the optimal points for the RP of the buds (Figure 22), which are set at the maximum levels of all factors, predicting an $EC_{50}$ of 0.16 mg/mL. Due to the low adjusted $R^2$ of the model, these points show low sensibility and thus might need further adjustments in future work, ideally using other varying factors. Still, it is convenient to remind that the minimize function was used due to the RP response being shown in $EC_{50}$ values, meaning that lower values represent higher antioxidant activity.

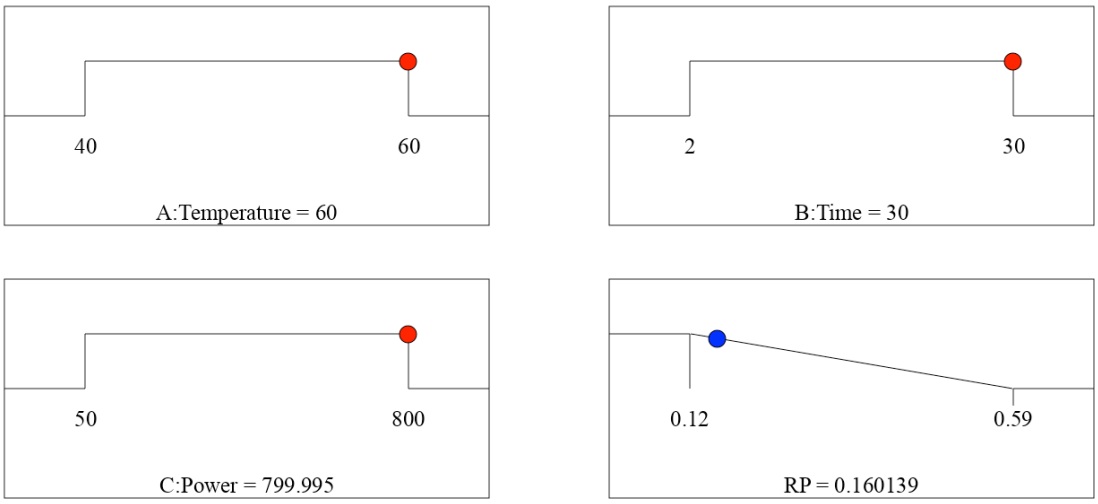


Figure 21. Optimal points and antioxidant activity prediction for the RP of the buds.

Due to the linear nature of the model, each factor was considered individually, and the plots are shown in figure 22. All three factors show a decreasing value over the increasing of the different values of each factor, and all three show a similar slope, except for microwave power, which shows a less steep slope.

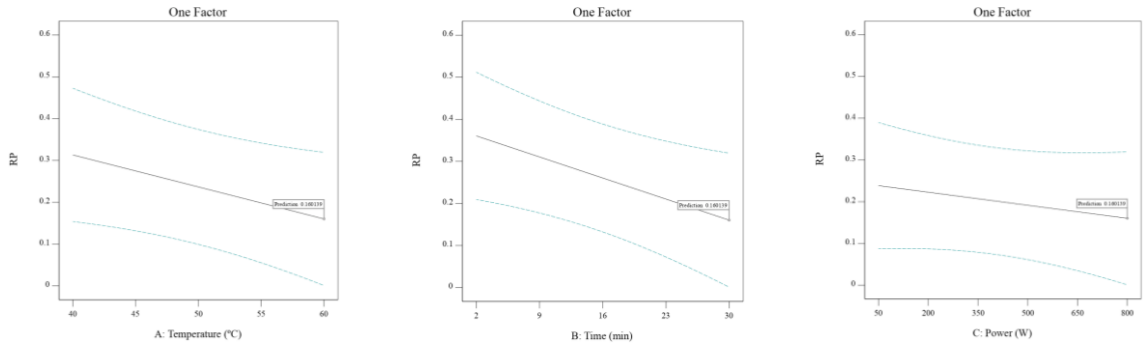


Figure22. Plots of the individual factors for the RP of the buds.

Although the model fitted the values, the adjusted R2 was low, and thus, the factors used to classify the response of RP were not the best. Still, the desirability function was used to obtain a response which included all three responses to point out the optimal point considering the dry yield, the $EC_{50}$ values of DPPH and RP, shown in figure 19.

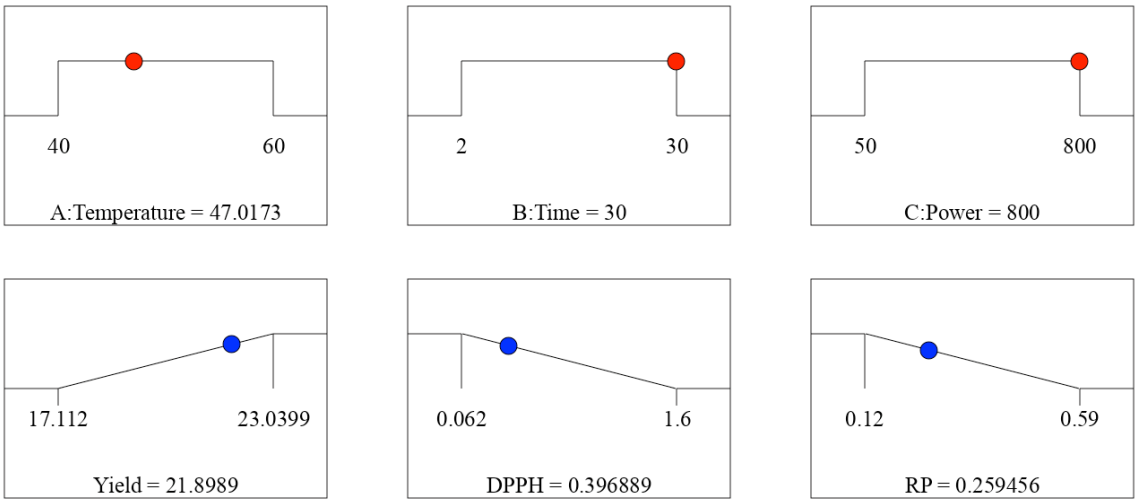


Figure 23. Plots of the desirability function showing the optimal points for all three responses for the buds.

The optimal points were set at 47 ºC, 30 minutes and 800W of microwave power, which are predicted to yield 23 g/100g of dry residue, 0.40 and 0.26 mg/mL of antioxidant activity for DPPH and RP, respectively. While there is a decrease from the obtained values for each response, the compounds obtained through this function are predicted to show activity against different radicals while yielding an acceptable dry residue. Still, due to the poor model obtained for the RP, in further studies, this assay could be swapped for another, with more sensitivity for the chosen factors, which could predict lower $EC_{50}$ values and higher dry yields.

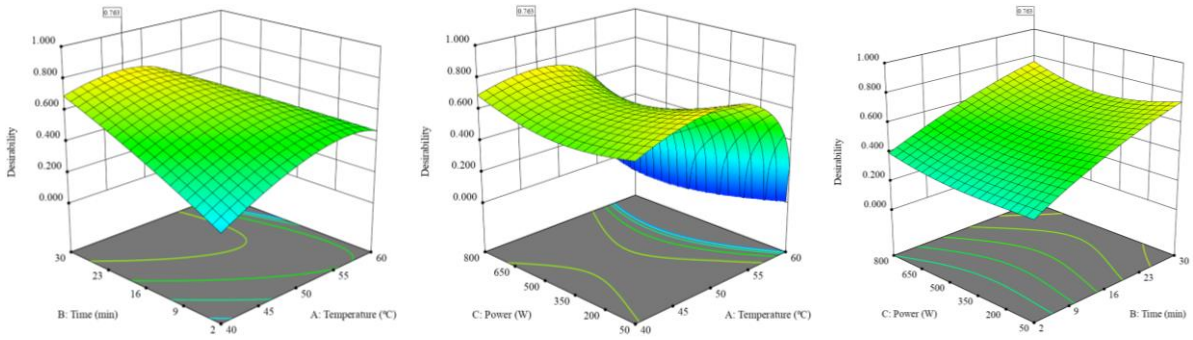


Figure 24. Plots of the response surface for the desirability of the buds.

Figure 24 shows the response surface methodology plots of the desirability for the three factors, pointing out that longer extraction periods and median temperatures promote the desirability (left plot), while power did not show much influence (right plot).

### V.7.2. Leaf optimization

Table 12 shows the experimental design for the extraction of the leaves, following the same format as table 11.

Table12. Experimental design of the MAE extraction of leaves.

| Run | Temperature (ºC) $\chi_1$ | Time (min) $\chi_2$ | Power (W) $\chi_3$ | Dry Yield (g/100g) $R_1$ | DPPH $EC_{50}$ $R_2$ | RP $EC_{50}$ $R_3$ |
|---|---|---|---|---|---|---|
| 1 | 60 | 2 | 425 | 17.813 | 0.8 | 0.33 |
| 2 | 50 | 2 | 50 | 17.504 | 0.57 | 0.24 |
| 3 | 40 | 30 | 425 | 20.659 | 0.87 | 0.36 |
| 4 | 50 | 30 | 50 | 22.470 | 0.66 | 0.283 |
| 5 | 50 | 16 | 425 | 21.137 | ~~0.24~~ | 0.34 |
| 6 | 50 | 2 | 800 | 18.584 | 1.02 | 0.34 |
| 7 | 40 | 16 | 50 | 18.163 | 1 | 0.28 |
| 8 | 50 | 16 | 425 | ~~23.048~~ | ~~1.89~~ | 0.37 |
| 9 | 50 | 16 | 425 | 20.828 | 0.63 | 0.374 |
| 10 | 40 | 2 | 425 | 16.971 | 0.58 | 0.329 |
| 11 | 60 | 16 | 800 | 21.767 | 0.82 | 0.36 |
| 12 | 40 | 30 | 800 | 23.942 | 0.62 | 0.27 |
| 13 | 50 | 16 | 425 | 20.638 | 0.59 | 0.345 |
| 14 | 40 | 16 | 800 | 20.117 | 0.88 | 0.33 |
| 15 | 60 | 16 | 50 | 22.725 | 0.26 | 0.27 |
| 16 | 60 | 30 | 425 | 26.355 | 1.1 | 0.26 |
| 17 | 50 | 16 | 425 | 20.970 | 0.41 | 0.2 |

Considering the leaves, the same factors and responses were analysed, using the same 17 run design. In terms of the first response ($R_1$), the dry yield, one outlier was found and removed from the data (run 8), and a significant two-factor interaction (2FI) model was obtained with a non-significant lack of fit, rendering a $R^2$ of 0.987 and an adjusted $R^2$ of 0.977. The coded equation was set at $Yield = 20.67 + 1.59 * \chi_1 + 2.82 * \chi_2 + 0.4437 * \chi_3 + 1.21 * \chi_1\chi_2 - 0.7279 * \chi_1\chi_3 + 0.0981\chi_2\chi_3$.

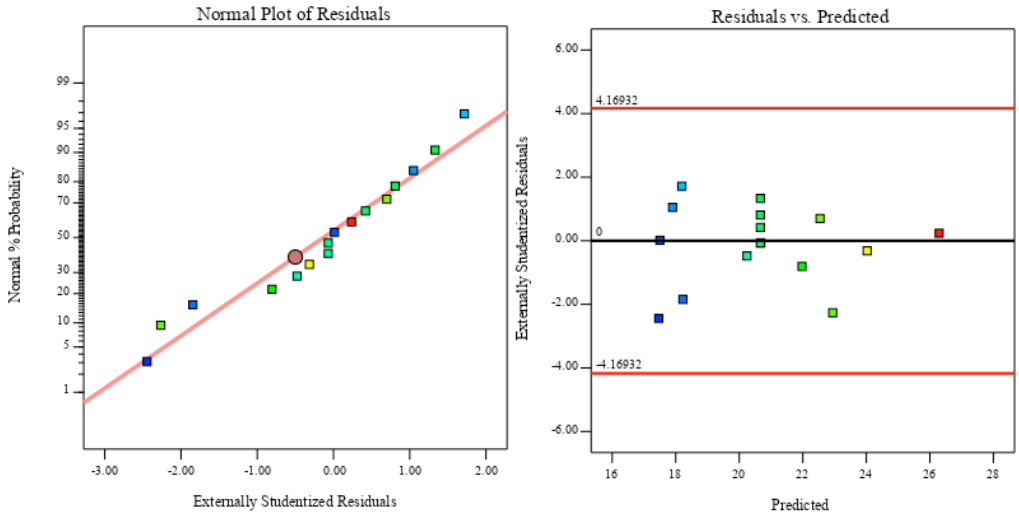


Figure 25. Plots of the individual factors for the yield of the leaves.

The plots of the residuals show a good dispersion over the trendline, and the residuals vs. predicted are within the upper and lower limits (Figure 25).

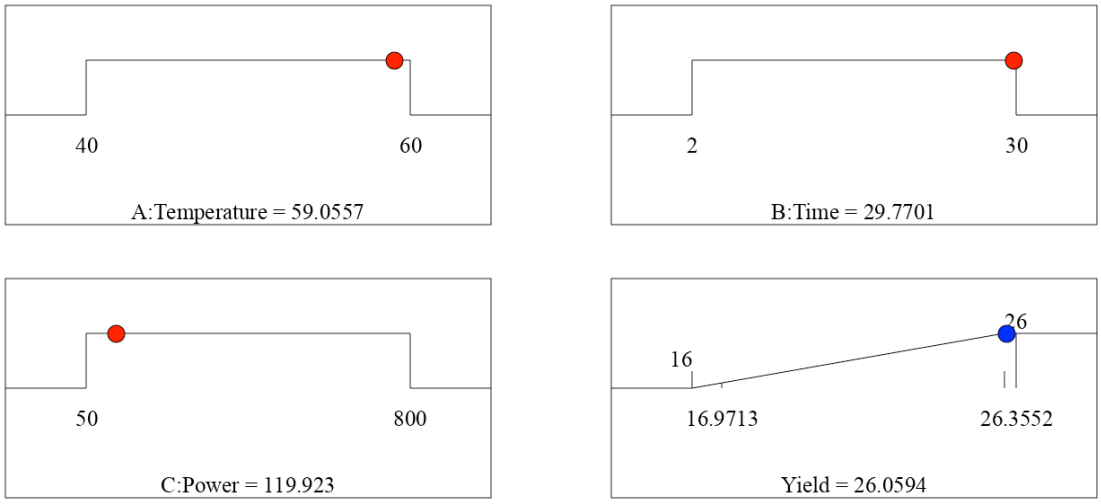


Figure 26. Optimal points and yield prediction of the optimal points for the leaves.

The optimal points (Figure 26) for the dry yield of the leaves were set at 59 ºC, 30 minutes and 119 W of microwave power, which are predicted to yield 26 g/100g of dry residue. Using the maximize function, the response surface plots (Figure 27) are show below, in which the left plot shows that higher yield is obtained at longer extraction times and higher temperatures, while the middle plot shows that higher temperatures when plotted with the microwave intensity reveal that the optimal point is near the central values.

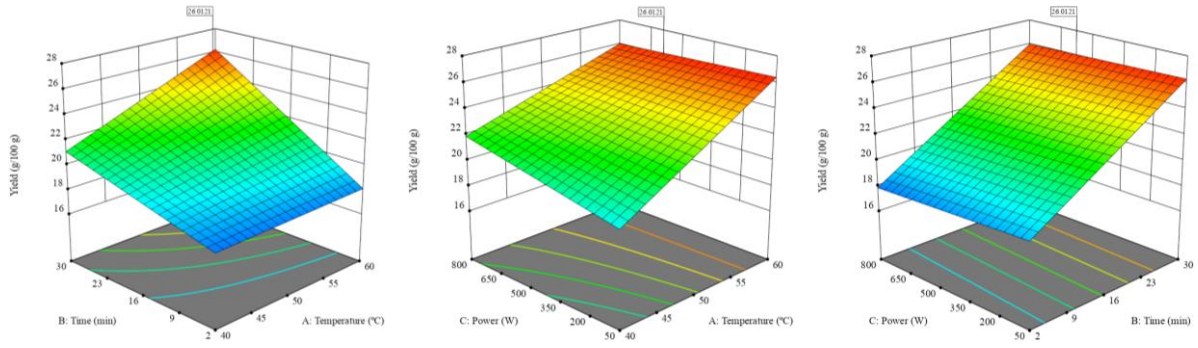


Figure 27. Plots of the response surface for the dry yield of the leaves.

Finally, the right plot shows the power and time, showing a linear interaction, in which the yield increases with time with little influence of the intensity of the microwaves. Overall, as like the model of the buds, the microwave has lower influence than time and temperature, which allows for considerable savings at an industrial level, using this technology at lower intensities for comparatively short periods (30 minutes) compared to other techniques.

Regarding the DPPH ($R_2$), response of the leaves, the selected model was a 2-factor interaction, after removing two outliers, showing the following coded equation $DPPH = 0.6613 \pm 0.1550 * \chi_1 \pm 0.0762 * \chi_2 + 0.1063 * \chi_3 - 0.2200 * \chi_1\chi_2 + 0.1700 * \chi_1\chi_3 - 0.1225\chi_2\chi_3$, with a non-significant lack of fit, a $R^2$ of 0.78 and an adjusted $R^2$ of 0.60.

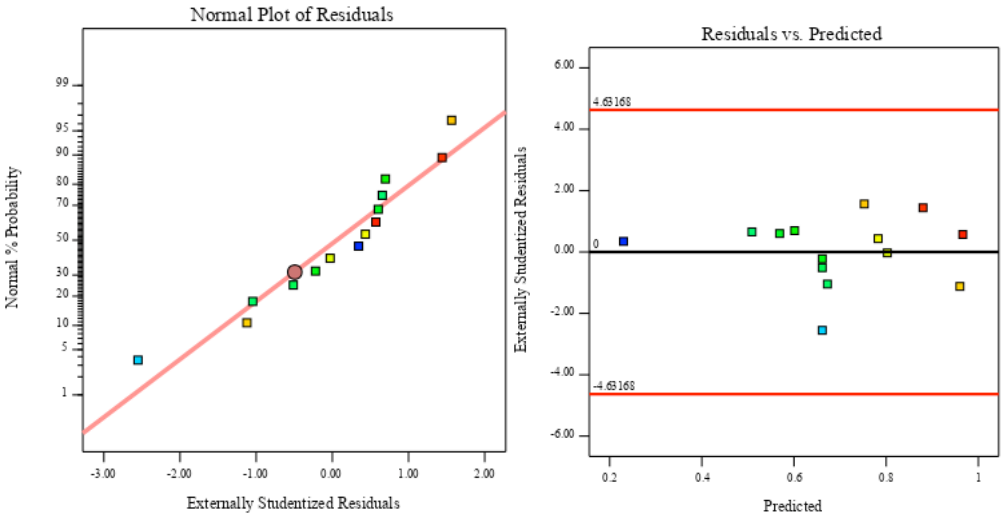


Figure 28. Plots of the individual factors for the DPPH of the leaves.

The plots of the diagnostics reveal a normal tendency with no residuals beyond the upper and lower limits (Figure 28), while the minimize function showed an optimal point at 59 ºC, 20 minutes and, once again, a low microwave intensity of 67 W, predicting an EC50 of 0.19 mg/mL (Figure 29).

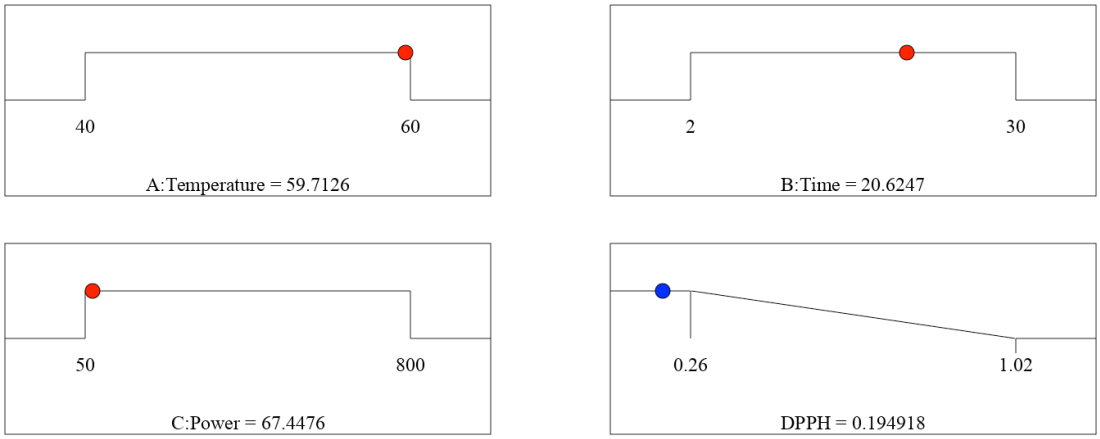


Figure 29. Optimal points and DPPH prediction of the optimal points for the leaves.

In Figure 30 the response surface plots show, in the left plot that longer extraction times and lower temperatures improve the antioxidant activity, while lower microwave intensities also have the same effect. The right plot shows that higher microwave intensities actually reduce the antioxidant activity, probably by destroying antioxidant molecules during the high intensity cycles.

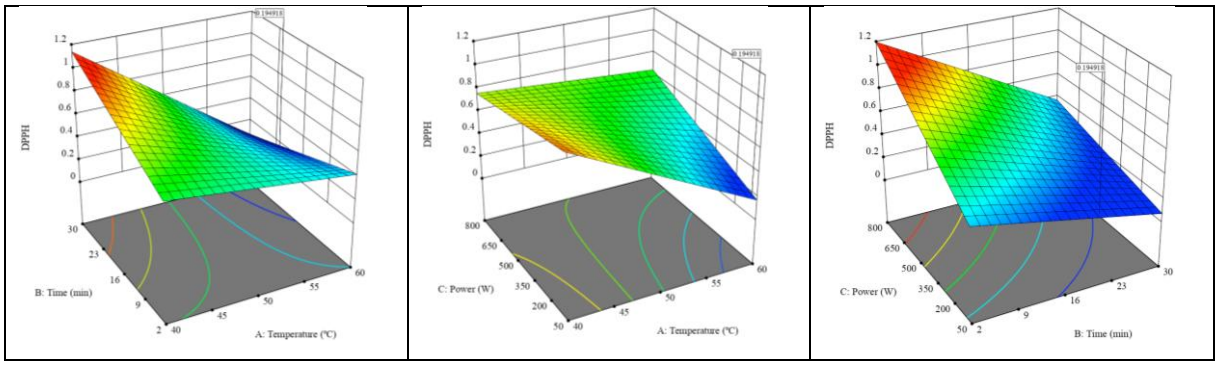


Figure 30 Plots of the response surface for the DPPH values of the leaves.

Once again, low values of microwave radiation, higher temperature and somewhat long periods of extraction promote the antioxidant activity through the DPPH assay.

Finally, for the RP assay, a quadratic model was defined, after deleting the last run which was an outlier. The $R^2$ was set at 0.89 and the adjusted $R^2$ at 0.73 with a non-significant lack of fit and a coded equation $RP = 0.3573 - 0.0099 * \chi_1 - 0.0083 * \chi_2 + 0.0284 * \chi_3 - 0.0252 * \chi_1\chi_2 + 0.0100 * \chi_1\chi_3 - 0.0283 * \chi_2\chi_3 - 0.0054 * \chi_1^2 - 0.0321 * \chi_2^2 - 0.0419 * \chi_3^2$.

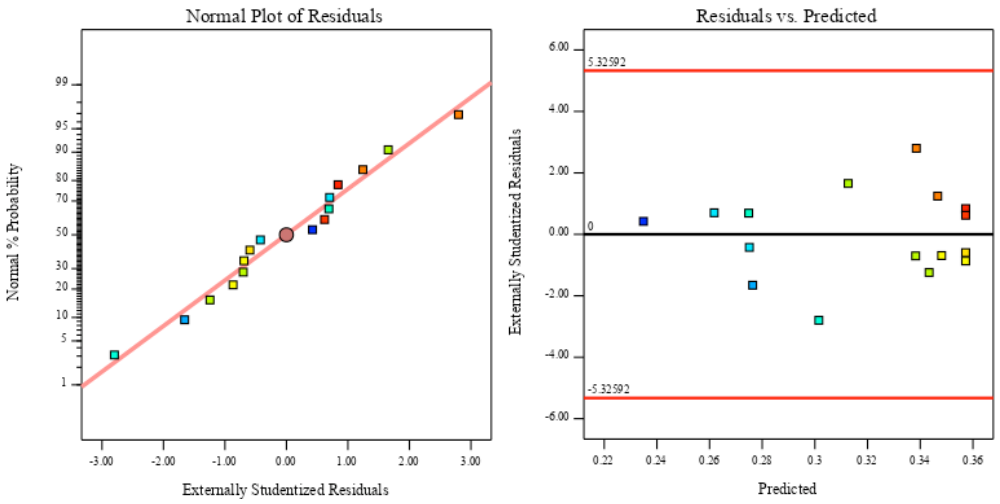


Figure 31. Plots of the individual factors for the RP of the leaves.

Regarding the diagnostics of the model, once again, all residuals were placed within the upper and lower boundaries, and the distribution was placed along the trendline. Using the minimize function, the optimal points were 44 ºC, 3 minutes and 53 W, predicting an $EC_{50}$ of 0.24 mg/mL, lower than any of the ones obtained in the 17 experimental runs. Of all the extractions, this one showed the least values of each factor.

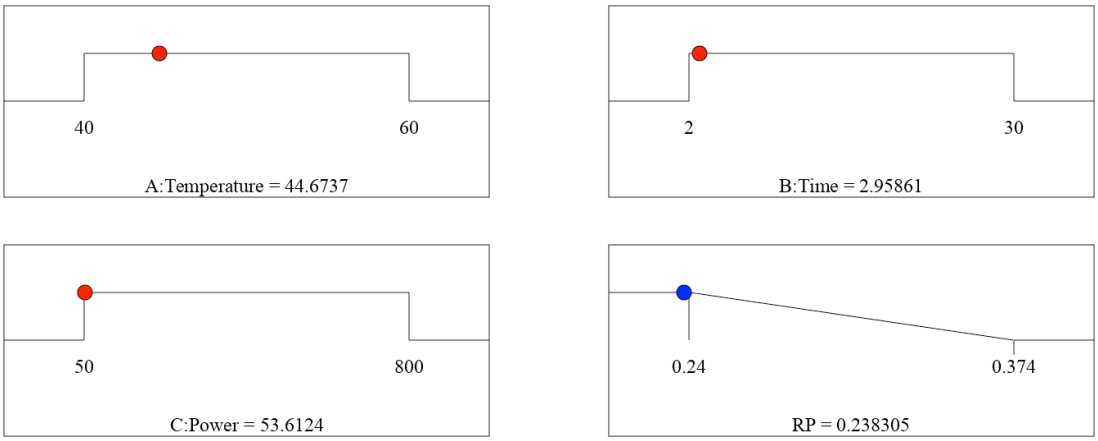


Figure 32. Optimal points and RP prediction of the optimal points for the leaves.

In figure 33, the three response surface plots show a quadratic effect, in which the left plot reveals that temperature had a low effect while the higher antioxidant activity was found for the least extraction time and tended to increase. The middle plot shows a considerable effect of temperature, with higher values promoting the $EC_{50}$ value, while low microwave intensity also promotes the activity. Finally, the right plot that shows power vs. time, shows that low power and time are the most effective in rendering high $EC_{50}$ values.

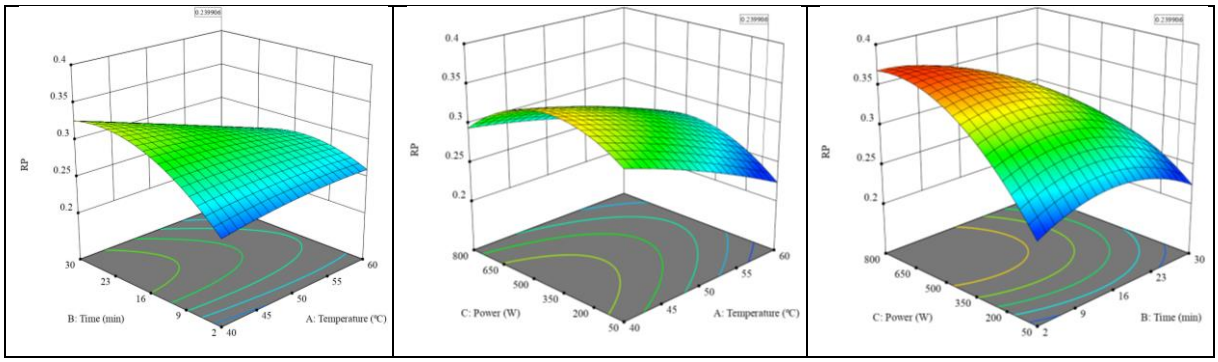


Figure 33. Plots of the response surface for the DPPH values of the leaves.

The desirability function, with maximize for yield and minimize for DPPH and RP, was used to find the optimal points that show the best points for all three responses, shown in figure 30.

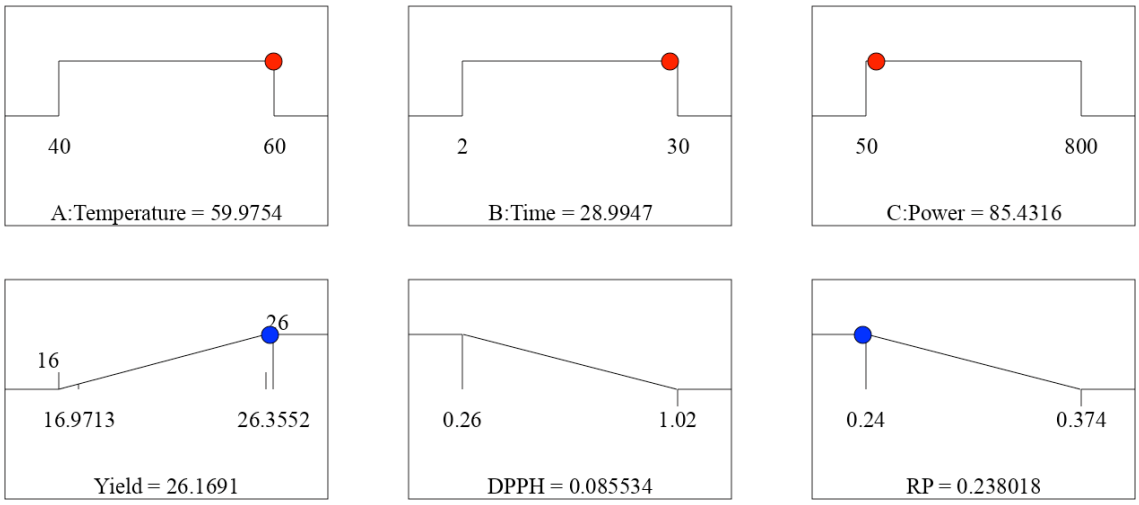


Figure 34. Plots of the desirability function showing the optimal points for all three responses for the leaves.

The desirability function defined the optimal points at 60 ºC, 29 minutes and 85W, predicting a dry yield of 26 g/100 g, an $EC_{50}$ of 0.08 mg/mL and 0.24 mg/mL for DPPH and RP, respectively. Interestingly, the prediction for all three responses was either above (for the yield) or below (for the $EC_{50}$) the runs. The response surface plots are shown in figure 31, showing the maximum desirability at the highest temperature and time (left), while microwave power promotes the desirability at the low intensities (middle).

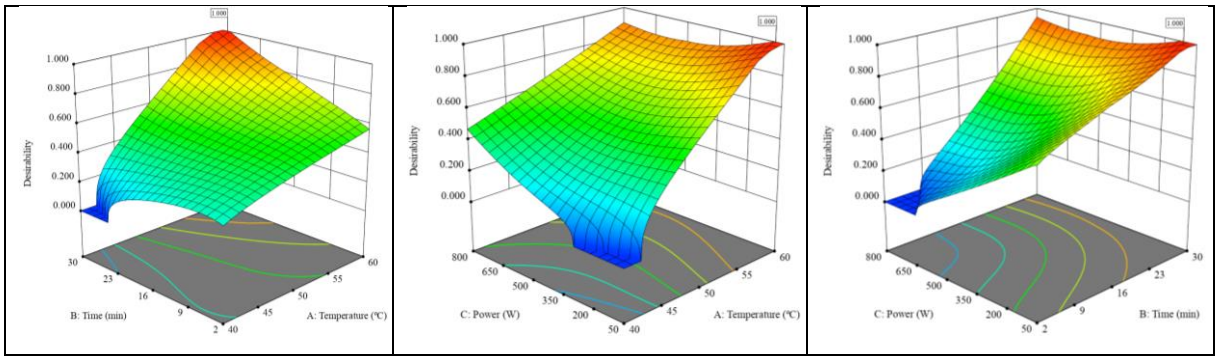


Figure 35. Plots of the response surface for the desirability values of the leaves.

Overall, considering all the extractions, it can be concluded that microwave extraction should be performed at low intensities, at high temperatures and medium intervals for the buds and leaves of *C. ladanifer*. Still, further analyses and factors should be considered to improve the realiability of the results.

## VI. Conclusions

Regarding the chemical composition of the essential oil from leaves and buds the major compounds indentified in the leaves were viridiflorol (13.2%), spathulenol (9.7%), 2,2,6-trimethylciclohexanone (5.2%), ledol (4.1%) and bornyl acetate (2.1%) while in the buds were viridiflorol (25.2%), ledol (10.4%), transpinocarveol (3.2%) bornyl acetate (3.0%) and 2,2,6-trimethylciclohexanone (2.4%). For the chemical composition of the concretes most of the compounds identified were not possible to be identified using the applied techniques. Nevertheless, in this work it was demonstrated that the concretes have antioxidant, antimicrobial and antifungal activities and therefore can be useful not only for cosmetic industries but potentially also for pharmaceutical industry. Also these results supports the use of this plant in traditional medicine as skin desinfectant.

To obtain rock-rose concretes, three different extraction methods were tested. In general, though the higher extraction yields obtained with Soxhlet extraction, microwave assisted extraction allowed to obtain similar extracts in terms of percentage of identified compounds and in general with higher amounts of interesting compounds such as viridiflorol, which is an odourant compound with floral scent. Furthermore, the soxhlet method requires a very long extraction time (6 hours), which comes at a huge expense in terms of energy, while the microwave extraction can be perceived as a « greener » technique since it requires much less extraction time. Finally, the use of response surface methodology demonstrated its usefulness for selecting better conditions aiming for the optimization of the extraction conditions.

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