**Universidade do Minho**
Escola de Engenharia

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Dissertação de Mestrado
Mestrado Integrado em Engenharia Biológica
Ramo de Tecnologia Alimentar e Química

Trabalho realizado sob a orientação do
**Professor Doutor António Augusto Martins de Oliveira Soares Vicente**

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# Sumário

**Extratos bioativos produzidos a partir de plantas medicinais tradicionais para aplicações alimentares e dietéticas**

A necessidade de uma dieta saudável tem estado cada vez mais presente na consciência das pessoas. Aliado a esta necessidade, surgiu também o crescente interesse em suplementos que possam atuar como uma ajuda adicional às necessidades fisiológicas dos consumidores, especialmente aqueles que são produzidos a partir de produtos de origem natural.

Assim, este trabalho teve como propósito, a produção de extratos naturais de plantas menos conhecidas, para aplicações dietéticas e nutracêuticas. Foram selecionadas 52 plantas de uma pesquisa generalizada sobre as suas características: atividades biológicas, solventes e condições de extração, grupos funcionais, fisiologia da planta, e as suas aplicações.

Assim sendo, as plantas foram testadas e analisadas quanto ao seu potencial antioxidante e grupos funcionais, em diferentes condições de extração e solventes. Desta análise extensiva, as plantas que apresentaram os melhores resultados foram: bétula branca, espinheiro marinho, rosa mosqueta, lúpulo e erva de S.João, todas com mais 70 % de inibição da oxidação. Os solventes que obtiveram os melhores resultados foram, de uma forma generalizada, os extratos de 40 % de etanol e os de 75 % de glicerol.

Posteriormente, procedeu-se a um processo de otimização das condições de extração e de valorização dos metabolitos das plantas, nomeadamente lúpulo e espinheiro marinho. Esta fase foi conduzida com a ajuda do programa Design Expert, sendo que apenas foi possível obter resultados precisos no caso do espinheiro marinho. Para os casos semelhantes ao do lúpulo, sugere-se uma redução das variáveis do modelo, de forma a melhorar a sua precisão.

**Palavras chave:** dieta saudável; suplementos; origem natural; plantas invulgares; antioxidante

# Abstract

**Bioactive extracts produced from traditional medicinal herbs to food and dietary applications**

The need for a healthy diet has been more present than ever in people's awareness. Aligned to this new drive, came along the increased interest of supplements as an additional help to support physiological necessities, especially those obtained from natural products.

This work has thus the purpose of producing natural based extracts from lesser-known plants, for dietary and nutraceutical applications. Accordingly, 52 herbs were selected from a generalized survey on their characteristics: biological activities, extraction solvents and conditions, functional groups, plants' physiology and applications.

Thus, the herbs were tested and analysed for their antioxidant potential and functional groups, on different conditions of extraction. From this extensive analysis, the herbs that presented the best results were: silver birch, sea-buckthorn, rose hip, common hop and St. John's wort, all with more than 70 % of antioxidant activity. The solvents which have obtained the best results were generally, the 40 % EtOH and the 75 % glycerol extracts.

Subsequently, an optimization process on the extraction conditions and metabolites' valorisation, was undertaken for common hop and sea-buckthorn. This phase was conducted with the help of the software Design Expert, nevertheless, a full analysis was possible only in the case of sea-buckthorn. For common hop (and similar situations), a reduction of the size of the model is suggested, as a means to improve its accuracy.



**Keywords:** healthy diet; supplements; natural based; lesser-known plants; antioxidant

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# List of Nomenclature

## Abbreviations

**GVR** – Grand View Research

**MI** – Mordor Intelligence

**CAGR** – Compound Annual Growth Rate

**EEA** – European Environment Agency

**EPA** – Environmental Protection Agency

**UN** – United Nations

**MEP** – methylerythritol 4-phosphate

**ODR** – Optical density reader

**DPPH** – 2,2-diphenyl-1-picrylthydrazyl

**DMSO** – Dimethyl sulfoxide

**E40** – Herbal extract of 40 % Ethanol

**E96** – Herbal extract of 96 % Ethanol

**G75** – Herbal extract of 75 % Glycerol

**Antio.** – Antioxidant

**NC** – Negative control

**AI** – Antioxidant inhibition

**BCM** – Bioactive compounds mass

**FP** – Flavonoids percentage

**SDE** – Software Design Expert

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# Introduction

## Context and Motivation

In today's society, the concern about what consumers eat in their meals is greater than ever. This new wave of nutrition awareness is making people (especially younger generations) much more predisposed to have a more healthy and natural-based diet (Sogari et al., 2018), which is changing the market demand. Practices such as veganism and vegetarianism took an immense impact on food industry. In recent past, several companies emerged for this new market and others adapted with the launch of several special segments (Saari et al., 2021). At this moment, it is safe to say that this is a trend that will not disappear in the future, and it could be a new standard practice for our nutrition (Saari et al., 2021).

Whenever eating is not enough considering our physiologic necessities, supplements can be an additional help. They came as a form of complementing/strengthening nutrition or general health (European Commission, 2002), and their interest grew alongside with the interest of a healthy diet, particularly in this pandemic time (Fortune Business Insights, 2021). Studies show that ≤ 75 % of individuals in developed countries consume one or more dietary supplements, (Barnes et al., 2016; Timbo et al., 2006; Bailey et al., 2013; Regan et al., 2011) which represents a high participation in these practices. With all of these motivations, market analysts forecast a steady growth for the next 7 years (Grand View Research, 2021; Mordor Intelligence, 2021; Fortune Business Insights, 2021), especially in Asia-Pacific, where people are culturally more open for these dietary changes.

Besides supplements' functions, consumers are progressively more cautious on their acquisitions, particularly regarding the source of supplements' ingredients (natural or chemical) (International Trade Centre, 2021). Therefore, botanical supplements acquired a higher demand in the

market, in terms of consumers' and producers' interest. In order to respond to this market, several companies started research programs to find natural sources of bioactive compounds, with attractive biological activities, high biological potential and possibility of industrialization.

In the same line, the aim of this work is the production of bioactive herbal extracts for a dietary/nutraceutical application.

## Goals

The purpose of this thesis is the production of extracts with a well-defined bioactive profile and a categorized composition.

To accomplish this purpose, the goals of this work are:

- Reviewing the state of the art regarding medicinal herbs, active substances, biological activities and medical/dietary applications;
- Production of extracts in different solvents and concentrations;
- Test of biological activity (antioxidant and if possible, inflammatory, cytotoxic);
- Quantification of flavonoids content;
- Optimization of extraction conditions for the most interesting herbs.

## Document Organization

This thesis is organized in two parts, Part I – State of the Art and Part II – Experimental Work. Part I is composed by four chapters, and Part II includes another three chapters.

Chapter 1 provides an overview of the global supplements market, in terms of sales, market growth, market shares, product and consumers segmentation, plant sources and consumers' demand. Chapter 2 presents a survey of the most interesting chemical groups and their sub-classification having in consideration their biological activities and

commercial applications. Chapter 3 introduces strategies of plant extraction, from the oldest to the most recent technologies, and Chapter 4 present some methodologies for the measurement of different antioxidant activities.

Part II is initiated by Chapter 5, with the presentation of all methodologies used in this work, such as plant preparation, plant extraction, purification process, biological activity assessments and optimization procedures. In Chapter 6, the results of the performed tests are presented and discussed. Finally, Chapter 7 includes the final takeaways of the work and future suggestions.

# Part I – State of the Art

# Chapter 1. Dietary Supplements

Supplementation is one of the easiest ways to improve health. Nowadays, it is really easy to find a supplement for virtually any problem, energy and weight management, immunity, cardiac health, diabetes, anti-cancer, general health, bone and joint health, gastrointestinal health and many more (GVR, 2021).

By definition, supplements are concentrated sources of nutrients or other substances that have a nutritional or physiological effect (including, but not limited to, vitamins, minerals, amino acids, essential fatty acids, fiber and various plants and herbal extracts), marketed in a "dose" (European Commission, 2002). This "dose" represents the supplements' format for a daily ingestion, having the possibility to be in soft gels, powders, tablets, capsules, gummies, liquids or even others (GVR, 2021), depending on consumers' taste.

## 1.1. Supplements Market

Dietary supplements are now a big part of people's diets. Studies report that ≤ 75 % of individuals in developed countries consume one or more dietary supplements (Barnes et al., 2016; Timbo et al., 2006; Bailey et al., 2013; Regan et al., 2011). These percentages display how currently, the general population is aware about their physiologic necessities and wellbeing. Along with the regular consumption of supplements and the new nutritional trends for a healthy diet, the pandemic situation also came to encourage the necessity of health prevention (MI, 2021). This was demonstrated by the surge in demand for the segments of digestive and immune health, in the recent past (MI, 2021). All of these signs reflect a healthy and prosperous market.

According to Grand View Research (GVR), the global dietary supplements market size was valued at USD 140.3 billion in 2020 with a compound annual growth rate (CAGR) of 8.6 % from 2021 to 2028 (GVR, 2021). The largest markets are North America and Europe, but the Asia-Pacific is the regional market with the highest growth rate (MI, 2021). In terms of product preferences, vitamins, botanicals, proteins and amino acids are the most requested, being the proteins the ones with the highest CARG, but in general every type of product expects a consistent growth (GVR, 2021). These are the ingredients in greater demand in order to support health conditions such as energy and weight management, general health, cardiac health and bone and joint health and others more (GVR, 2021; GVR, 2020). In this market, the energy and weight management, is the main sector of the market, helping to solve health problems such as cardiovascular diseases, cancer, and diabetes that are consequences of sedentary lifestyle and changing in food habits in developed countries (GVR, 2020). For the type of consumption, there is a clear favouritism for tablets and capsules, covering more than 50 % of the global market (GVR, 2021).

### 1.1.1. Supplements Market: Botanicals

Botanicals are the second category of products most wanted in the supplements market (GVR, 2021). They are defined as preparations obtained through solvent extractions from plants and are one of the oldest forms of supplementation and medicine (Food Processing, 2012). This type of supplements has been gathering a lot of supporters recently, especially veganism and vegetarianism supporters since the ingredients are all plant-based (GVR, 2021; GVR, 2020).

According to GVR, this market segment was valued at USD 27.47 billion in 2020 and it is expected to grow at a CAGR of 9.1 % until 2028 (GVR, 2021). This forecast represents the increased interest in this renewed market, but everything points to an even higher expansion of the market

since various producers continue to innovate on the production and application of botanicals (GVR, 2021).

In terms of market drives, both the botanical and global markets present various similarities. The best regional markets still are the North America and Europe, but the one with greater CARG is the Asia-Pacific by the same reasons as above (GVR, 2020). For the type of consumption, the consumers still maintain the tablets and capsules preference, since it is the easiest way to do it.

#### 1.1.1.1. Botanicals (Plants sources)

As already mentioned, botanicals are one of the oldest forms of supplementation and medicine in human history. The oldest written evidence of medicinal plants' use has been found approximately 5000 years ago (Petrovska, 2012), being its real first use even older. In terms of plants' selection, it varies greatly across the geography and the cultures of the time, as demonstrated in the numerous books recovered from the past, such as the Chinese book on roots and grasses "Pen T'Sao" (2500 BC), the Indian holy books "Vedas" (2000 BC), the "Ebers Papyrus" (1550 BC) and many others through human history (Petrovska, 2012). This data collection was extremely important for the survival of those times and the near future, continuing until these days, with the current pharmacopeias and herb monographs. With all of this accumulated knowledge was possible to treat and prevent several illnesses and injuries, but the research still continuous since there is a considerable number of plants yet to be studied and discovered.

In market terms, there are herbs more interesting than others. Currently, the most commercialized herbs are the ones that are capable of treating the most common problems, such as general health, energy and weight management, immunity and many others (GVR, 2021). From the perspective of consumers' preference, there is a clear favoritism for some specific herbs in the last few years such as, ginkgo, green tea, artichoke, ginseng, aloe vera, echinacea, cranberry and many more (Garcia-Alvarez et al., 2014; Gray, 2015; Loria, 2019). However, this favoritism is quite

unstable in terms of sales volume, being sometimes difficult for business owners decipher future trends/demands. Therefore, the main drive of this type of market is to find better plants sources, with high biological potential and interesting biologicals activities to solve current health problems more effectively or to help solving emerging ones (Chau et al., 2011).

In this line, the present work started by conducting a generalized survey of less known/commercialized herbs. This research had the purpose to recognize the extraction parameters, parts used, typical applications, bioactive compounds and biological activities of a selection of 52 herbs. The list of those herbs is presented in Table 1 and Figures 1 and 2 exemplify the structure and the content of this database.

## 1.2. Regulation and Safety

To date, dietary supplements are classified as “food” that has the purpose to complement some type of deficiency in our diet or to improve any health condition by stimulating the physiologic response (European Commission, 2002). This classification is given by the European Commission, but several governments or regulatory agencies have their own definition, which poses a coherence problem to consumers in the international market (Thakkar et al., 2020). For example, green tea extract is classified as “Medicines” in Canada and Australia while being classified as “Dietary Supplements” in Japan, China, New Zealand, and the EU (Thakkar et al., 2020). However, it is treated as both a botanical drug for topical use and as a supplement in the US (Thakkar et al., 2020). This incoherence can lead to a serious health problem, since a product regulated as “food” cannot have therapeutic claims, but a product regulated as medicine can have therapeutic claims (Thakkar et al., 2020), which can induce an inadequate consumption of supplements.

In terms of safety, it is really important to know exactly what we are consuming. Industries, such as pharmaceuticals and cosmetics, are obliged to implement various analytical methods to verify and test the composition and safety of their products. However, unlike drugs and

cosmetics, supplements do not require safety tests to ensure that the substances in the supplements have not dangerous interferences with the human body or other substances already consumed (Cassileth et al., 2009). This represents a serious hazard for human health, especially for cancer patients, where the efficiency of the chemotherapy drugs could be affected (Cassileth et al., 2009)

Table 1. List of herbs, associated code and the respective part used

| Code Name | Latin Name | English Name | Part used |
|---|---|---|---|
| 1 | *Arcticum lappa* | Dried burdock | Roots |
| 2 | *Elymus repens* | Couch grass | Rhizome |
| 3 | *Betula pendula* | Silver birch/ Warty birch | Leaves |
| 4 | *Hippophae rhamnoides* | Sea-buckthorn | Fruits |
| 5 | *Fructus cynosbati* | Rose hip plant | Fruits and seeds |
| 6 | *Trifolium pratense* | Red clover | Flowers |
| 7 | *Malva sylvestris* | Common mallow | Stems and leaves |
| 8 | *Sambucus nigra* | Elderberry | Fruits |
| 9 | *Galega officinalis* | Galega/Goat's-rue | Stems and leaves |
| 10 | *Solidaginis virgaureae* | European Goldenrod | Stems and leaves |
| 11 | *Crataegus monogyna* | Hawthorn | Flowers and leaves |
| 12 | *Achillea millefolium* | Yarrow | Stems and leaves |
| 13 | *Fragaria vesca* | Wild Strawberry | Leaves |
| 14 | *Calluna vulgaris* | Heather | Stems, leaves and flowers |
| 15 | *Taraxacum officinale* | Dandelion | Roots |
| 16 | *Saponaria officinalis* | Common Soapwort/ Bouncing bet | Roots |
| 17 | *Humulus lupulus* | Common hop | Flowers |
| 18 | *Allium sativum* | Garlic | Bulbs |
| 19 | *Tilia cordata* | Small-leaved lime | Leaves, fruits and flowers |
| 20 | *Melilotus officinales* | Yellow sweet clover/Yellow melilot | Stems and leaves |
| 21 | *Artemisia absinthium* | Wormwood | Stems and leaves |
| 22 | *Hypericum perforatum* | St John's-wort | Stems and leaves |
| 23 | *Taraxacum officinale* | Dandelion | Leaves |
| 24 | *Leonurus cardiaca* | Motherwort | Stems and leaves |
| 25 | *Symphytum officinale* | Common comfrey | Roots |
| 26 | *Bellis perennis* | Daisy | Flowers |
| 27 | *Vaccinium Vitis-idaea* | Lingonberry/Mountain cranberry | Stems and leaves |
| 28 | *Urtica dioica* | Urtica | Leaves |
| 29 | *Galeopsis tetrahit* | Hemp Nettle | Stems and leaves |
| 30 | *Ocimum basilicum* | Basil | Stems and leaves |
| 31 | *Allium ursinum* | Ramson, Wild garlic | Stems and leaves |
| 32 | *Veronica officinalis* | Veronica | Stems and leaves |
| 33 | *Glechoma hederacea* | Ground-ivy | Stems and leaves |
| 34 | *Agrimonia Eupatoria* | Common Agrimony | Stems and leaves |
| 35 | *Valeriana officinalis* | Valerian | Roots |
| 36 | *Marrubium vulgare* | White horehound | Stems and leaves |
| 37 | *Genista tinctoria* | Dyer's greenweed | Stems and leaves |
| 38 | *Glycyrrhiza glabra* | Liquorice | Roots |
| 39 | *Equisetum arvense* | Horsetail | Stems and leaves |
| 40 | *Fagopyrum esculentum* | Buckwheat | Stems and leaves |
| 41 | *Calendula officinalis* | Calendula | Flowers and sepal |
| 42 | *Salvia officinalis* | Sage | Stems and leaves |
| 43 | *Thymus serpyllum* | Breckland thyme | Stems and leaves |
| 44 | *Origanum vulgare* | Oregano | Stems and leaves |
| 45 | *Salix alba* | Willow | Crust/peel |
| 46 | *Salvia rosmarinus* | Rosemary | Leaves |
| 47 | *Matricaria chamomilla* | Chamomile | Flowers |
| 48 | *Sambucus nigra* | Elder flower | Flowers |
| 49 | *Teucrium chamaedrys* | Wall germander | Stems and leaves |
| 50 | *Sinapis alba* | White Mustard | Seeds |
| 51 | *Cichorium intybus* | Common chicory | Roots |
| 52 | *Silybum marianum* | Milk thistle | Fruits |

# Rose hip

*Rosa Canina (Fructus cynosbati)*

**Czech Name**

Šípek drcený s jádry

**English Name**

Rose hip, Dog rose

**Part Used**

Fruits and seeds

**INCI**

Rosa Canina fruit extract

**Extraction conditions**

Tincture (DER 1:2), Solvent: Ethanol 70 % (w/w) for 15 weeks at 23 ± 2 °C (Polar compounds);

Powder substance (DER 1:10), Solvent: Methanol 80 % with 0.5 % trifluoroacetic acid with stirring at ambient temperature;

Liquid extract (DER 4:1), Solvent: methanol/water for 3 days constantly shaken at 120 rpm/min at room temperature.

**Bioactive Compounds**

Rich in polyphenolic compounds, including flavonoids (anthocyanins, procyanidins, catechin, quercetin), phenolic acids (gallic and ellagic acids), fruit acids (malic acid, citric acid), kaempferol, apigenin, resveratrol. Valuable source of vitamin C, A, $B_1$, $B_2$, $B_3$, and K, carotenoids (including lycopene, β-carotene, zeaxanthin), tocopherols, volatile oils and minerals (potassium and phosphorous but mostly calcium).

**Biological Activity**

Antioxidant, anti-inflammatory, antibacterial, antimutagenic, anticancerogenic, anti-ulcerogenic, antinociceptive, anti-obesity, anti-diabetic and antiproliferative.

Figure 1. Detailed information of the herb Rose hip Part 1.

**Typical Application**

Prevention and therapy of common cold, flu, and other infections, gastrointestinal, kidney and lower urinary tract disorders, diabetes, arthritis, lung ailments, treatment of various inflammatory diseases and immunity deficiencies.

**Notes**

Nothing to add.

**References**

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Figure 2. Detailed information of the herb Rose hip Part 2.

Besides supplements' composition, the safety of dietary supplements depends largely on the dose (Dwyer et al., 2018). High doses of some substances can lead to the development or aggravation of several medical issues, for example, the consumption of high doses of vitamin D, may cause adverse effects in the liver function of a normal person (Rooney et al., 2017). There are reports in the literature of several other cases with mild and severe reactions to specific substances (Drüeke, 2012) or to complex mixtures of herbal extracts (Brown, 2016), thus reinforcing the idea of a lack of information about the safe levels of intake of non-nutrient bioactives in supplements (Dwyer et al., 2018). This is a very alarming situation in terms of human health since no prescription is required to purchase any of these products (Dwyer et al., 2018).

Thus, for some authors, the optimal attitude to this matter is to always consult a physician or other medical professionals' opinion before consuming any type of supplement, because even with an informed opinion this topic is very ambiguous (Cassileth et al., 2009).

# Chapter 2. Bioactive compounds

Plants are the source of an outstanding spectrum of substances, that can reach between 200,000 to 1,000,000 metabolites in the plant kingdom (Saito, 2010). It is well known in the scientific community that they are one of the best chemicals systems/factories available and have a remarkable chemical library (a great part still unknown) (Durazzo et al., 2018) for any kind of health application (nutritional or pharmaceutical) or even other industrial segmentations (cosmetic, chemical, energy, etc.). This notion encouraged even more the companies and organizations to search and research new natural compounds, with the straight purpose of creating value and knowledge. However, its valorisation is not just within the economic insights, the use of natural compounds also came as an environmental alternative to the substances derived from chemical synthesis.

## Background

In plants' life, several substances are produced, each type with their particular purpose. From this consortium of compounds, two groups can be established: the primary and the secondary metabolites. The primary metabolites are labelled as chemical substances aimed for growth and development of the organism, such as proteins, lipids, carbohydrates and amino acids (Azmir et al., 2013). The secondary metabolites, on the other hand, are substances that have no use in the growth phase and usually are produced in a sub-sequent phase of the development of the plant (Azmir et al., 2013). Therefore, the purpose of these compounds is more directed to the survival ability of the plants in order to overcome local challenges, being these substances a way of interacting with their surroundings (Azmir et al., 2013). The respective compounds can be categorized in various classes

and subclasses, being quite difficult to present a standard definition since, various authors present their positions, none until today universal. According to Kutchan et al. (2015) the bioactive compounds (the secondary metabolites) are composed of four main categories: terpenoids, alkaloids, phenolics and cyanogenic glucosides and glucosinolates (this last category it was not approached in this work given its toxicity, lower information/research about its safety and little biotechnological applications (Schrenk et al., 2019)). This categorization is technically based on their chemical structures and their variations through the respective subclasses.

In terms of substances' presence, it varies greatly, and they can be found in every part of the plants' structure, for example, in the roots, leaves, stems, flowers, fruits, bulbs, seeds and barks (Petrovska, 2012). For this reason, it is necessary to trace where the compounds of interest have their biggest presence, in order to achieve the best valorization.

## 2.1. Terpenoids

Terpenoids are an assembly of more than 30000 molecules described to date, being the largest class of natural compounds (Kutchan et al., 2015a). It is a group with a wide variety of structures, all based in the same biosynthetic origin, the five-carbon isopentane units or in a more current form, isoprenoids or terpenes (Puri, 1998a). Terpenes have the molecular formula of $C_5H_8$ , and most of the other terpenes are variations of this base, such as monoterpenes, $C_{10}$; sesquiterpenes, $C_{15}$; diterpenes, $C_{20}$; sesterterpenes, $C_{25}$; triterpenes, $C_{30}$; and so on (Zhao et al., 2015). An easier way of representing terpenes is through the number of isoprene units represented by n, $(C_5H_8)_n$(Zhao et al., 2015).

For the biosynthesis of this basic structure, two pathways can be chosen, the mevalonate pathway or the methylerythritol 4-phosphate (MEP) pathway (Yang et al., 2016). The MEP pathway is an exclusive chemical path of the plant kingdom and proved to be a great advantage since with

this possibility the production of terpenoids became more efficient (Kutchan et al., 2015a). The nature of this efficiency comes from the production optimization, where the chemical pathways contribute differently to the biosynthesis of terpenoids (Kutchan et al., 2015a). For instance, the mevalonate pathway supplies most of $C_5$ units for the biosynthesis of $C_{15}$ (sesquiterpenes), $C_{30}$ (triterpenes and sterols), and larger compounds, whereas the MEP pathway supplies most of $C_5$ units for the biosynthesis of $C_{10}$ (monoterpenes), $C_{20}$ (diterpenes), and $C_{40}$ (carotenoids) compounds (Kutchan et al., 2015a). On the second phase of terpenoids biosynthesis, it is involved the fusion of $C_5$ units enabled by condensation, although for the production of major substances ($C_{30}$ and $C_{40}$ terpenoids) this addiction process is not employed (Kutchan et al., 2015a). For the assembly of this type of structures, for example, to create a $C_{30}$ compound two $C_{15}$ compounds are merged, continuing to even bigger substances as needed (Kutchan et al., 2015a). Even if these two metabolic pathways are physically separated the spatial exchange of intermediates (isopentenyl diphosphate or dimethylallyl diphosphate) between the two pathways is widely documented in scientific community, being one of the assumptions why plants can produce such a variety of terpenoids metabolites (Kutchan et al., 2015a).

### 2.1.1. Roles in plants

Terpenoids have numerous survival and defense functions. Volatile terpenoids, for example are constituents of floral scents and act in the attraction of pollinators, or in other cases in a defensive position where the emission of (E)-β-caryophyllene act against microbial pathogens (e.g., *P. syringae*) (Tholl, 2015). This defensive action is also reported in interactions with animals where the emission of monoterpenes from the leaves have prevented herbivores to eat them (Tholl, 2015). This behaviour is well documented, even with other types of predator-prey interactions, but as well in beneficial interactions, either in an aerial or subterranean area.

Besides terpenoids being bioactive compounds, some of them are aligned to the basic plant growth and development, being classified as well as primary metabolites (Kutchan et al., 2015a). Sometimes the same component can have a dual functionality, depending on whether the stimulus is internal or external, as for example, the carotenoids participate in the energy-transfer processes of photosynthesis or in the protection of photosynthetic tissues from oxidation, and in the other spectrum, attract animals to flowers and fruits for dispersal of pollen and seeds (through pigmentation as a visual effect) (Kutchan et al., 2015a). Being literature of terpenoids quite extensive, there are several other examples of compounds acting in the growth and development of plants as examples of terpenoids functionality.

### 2.1.2. Roles in humans

In a human society the application of these substances is really diversified. Firstly, from the intense study made in plants functionality, it was possible to understand the crucial role of this compounds on the surviving ability of plants. In this view, the application of these substances in protecting and stimulation of agriculture, as herbivore deterrents, pest repellents, germination and growth inhibitors of competing plants and pollinator presence enhancers was evident, in addition to the fact that these compounds have a low toxicity to mammals and lack of persistence in the environment (Kutchan et al., 2015a).

In a more industrialized application, terpenoids are used as flavors and fragrances of foods, beverages, soaps, perfumes, toothpaste, and other products (Kutchan et al., 2015a). From a health point of view, terpenoids also have their contributions, since many of them have a nutritional significance, having regard to vitamins A, D, E, and K, entering the nutraceutical segmentation (Kutchan et al., 2015a; Zhao et al., 2015). Moreover, in the pharmaceutical field, there are many examples of the use of terpenoids, for instance, on the treatment cancer, inflammation, viral

(e.g., SARS-corona virus) and bacterial infections, cardiac insufficiency, malaria, as well as several other cases available in literature (Teoh, 2016; Zhang et al., 2005; Kutchan et al., 2015a).

## 2.2. Alkaloids

Alkaloids are mainly present in plants; having more than 12,000 compounds been identified, they are not exclusive to them (Ali et al., 2019). There are other organisms (animals, fungi and bacteria) that also produce alkaloids, in most of the cases as a protective measure (Zhao et al., 2015).

Their constitution usually contains a nitrogen atom as part of the heterocyclic ring, an amine functional group (Teoh, 2016). Their biosynthesis in plants is normally performed by simple precursors and unique enzymes (Kutchan et al., 2015b). For this reason, it is unlikely to present a specific pathway, since there are so many chemical variations in order to produce alkaloids. There are in fact some group variations, where alkaloids combine with steroidal, secoiridoid (e.g., secologanin) or other terpenoid-type moieties (Kutchan et al., 2015b). In general, most of alkaloid's production is based in the modification of amino acids, mainly decarboxylation or deamination, that undergoes into further steps such as methylation, hydroxylation and oxidation (Yang et al., 2016).

Based on their chemical structure (ring system presented) and on biosynthetic origin of the proteins and amino acids, alkaloids can be classified into fourteen groups (Puri, 1998b). However, given the immense structural diversity, some compounds do not fit in any group or fit more than one, being a challenge to create any kind of categorization (Puri, 1998b).

### 2.2.1. Roles in plants

The main objective of alkaloids is directed to plant protection. There are some studies approaching alkaloids as growth regulators and

stimulators, but only in alkaloid-rich plants, since alkaloids inserted in alkaloid-poor plants actually inhibited their growth, being allelopathic (Waller et al., 1978). There is also reference that alkaloids are a storage form of nitrogen or substitutes for plants in minerals, but poorly supported (Waller et al., 1978; Puri, 1998b). Therefore, the leading reason for the production of this substances is direct to the defence against any kind of threat. There are several examples portraying these interactions, for instance, in the plant family of *Nicotiana sp.* the nicotine acted as a toxin for the insects, being this substance one of the first insecticides used by humans (Kutchan et al., 2015b). Another classical example is the caffeine found in the seeds and leaves of cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), maté (*Ilex paraguariensis*) and coffee beans (*Coffea arabica*) that defended these plants against the infestation of insects and larvae (Kutchan et al., 2015b). In general, this is the main role of alkaloids in plants, several other examples are available in literature highlighting the same purpose, but against different protagonists such as insects, herbivores, fungi, bacteria and virus (Waller et al., 1978; Kutchan et al., 2015b).

### 2.2.2. Roles in humans

Alkaloids have been present in human society even before they were officially discovered (Ali et al., 2019). For a long time, they have brought good, through the ancient medical recipes in order to cure or help a very wide range of ailments, or bad, through the several narcotics that have trapped people to addiction (Teoh, 2016). There are several examples of drugs abuse such as nicotine, cocaine, heroin, and other opium derivatives that influenced a lot of individuals' lives, but as well geopolitical implications along human history (Teoh, 2016). As mentioned earlier, they also brought many benefits, with a special focus to pharmacology. With scientific progression, the study of alkaloids has greatly increased, since they presented some many interesting activities that with the right preparation and application could immensely improve human lives. Table

2 shows some examples of these medical contributions, and with continuous research, more will appear (Kutchan et al., 2015b). Despite of having other applications for alkaloids, for example in agriculture, the main use and research still is directed to pharmacy.

Table 2. Physiologically active alkaloids used in modern medicine (adapted from Kutchan et al., 2015b)

| Alkaloid | Plant source | Use |
|---|---|---|
| **Ajmaline** | *Rauwolfia serpentina* | Antiarrhythmic that functions by inhibiting glucose uptake by heart tissue mitochondria |
| **Atropine, (±) hyoscyamine** | *Hyoscyamus niger* | Anticholinergic, bronchodilator |
| **Caffeine** | *Coffea arabica* | Widely used central nervous system stimulant |
| **Camptothecin** | *Camptotheca acuminata* | Potent anticancer agent |
| **Cocaine** | *Erythroxylon coca* | Topical anesthetic, potent central nervous system stimulant, and adrenergic blocking agent; drug of abuse |
| **Codeine** | *Papaver somniferum* | Relatively nonaddictive analgesic and antitussive |
| **Coniine** | *Conium maculatum* | First alkaloid to be synthesized; extremely toxic, causes paralysis of motor nerve endings, used in homeopathy in small doses |
| **Emetine** | *Uragoga ipecacuanha* | Orally active emetic, amoebicide |
| **Morphine** | *Papaver somniferum* | Powerful narcotic analgesic, addictive drug of abuse |
| **Nicotine** | *Nicotiana tabacum* | Highly toxic, causes respiratory paralysis, horticultural insecticide; drug of abuse |
| **Pilocarpine** | *Pilocarpus jaborandi* | Peripheral stimulant of the parasympathetic system, used to treat glaucoma |
| **Quinine** | *Cinchona officinalis* | Traditional antimalarial, important in treating *Plasmodium falciparum* strains that are resistant to other antimalarials |
| **Sanguinarine** | *Eschscholzia californica* | Antibacterial showing antiplaque activity, used in toothpastes and oral rinses |
| **Scopolamine** | *Hyoscyamus niger* | Anticholinergic, effective against motion sickness |
| **Strychnine** | *Strychnos nux-vomica* | Violent tetanic poison, rat poison, used in homeopathy |
| **(+) - Tubocurarine** | *Chondrodendron tomentosm* | Nondepolarizing muscle relaxant producing paralysis, used as an adjuvant to anesthesia |
| **Vincristine** | *Catharanthus roseus* | Antineoplastic used to treat childhood leukemia and other cancers |

## 2.3. Phenolics

Phenolic compounds are probably the most diversified group of secondary metabolites. Aside from all conformational variations, most phenolic compounds have in their structure an aromatic ring and a hydroxyl group attached (Zhao et al., 2015). This simple structure it is termed as phenol and is the simplest phenolic compound. The rest of the phenolic compounds will be variations of this base, depending on which metabolic pathway is followed (Babenko et al., 2019). For a wide variety of compounds, it comes a great variety of metabolic construction, therefore phenolics probably have the highest number of different construction paths, such as the shikimic acid, phenolic acids, the phenylpropanoids, the phenylpropanoid-acetate (phenylpropanoids pathway variation) and the acetate-melonate pathways (Babenko et al., 2019; Kutchan et al., 2015c). Initially, phenolic compounds are synthesized through shikimate and acetate-malonate pathways. Along with these pathways, as demonstrated in Figure 3, the metabolic pathways connect between each other, enabling the creation of structures even more complex (Babenko et al., 2019). For example, coumarins, are formed because the shikimate pathway provides the synthesis of phenylalanine aromatic amino acid for the key enzyme of the phenylpropanoid pathway – phenylalanine ammonia-lyase (PAL) (Babenko et al., 2019).

Given all the metabolic possibilities, several major classes were described, each one with its variations (subgroups). Depending on the authors many categorizations were proposed but having in regard the basic skeleton (chemical formula (e.g., $C_6C_3C_6$)) and the basic structure (2D representation) there are sixteen major groups: simple phenols, phenolic acids, coumarins, stilbenes, lignins, flavonoids, etc. (Lourdes, 2013). Flavonoids, of all is probably the most interesting class, since it is the most populated group in phenolics aggregation. Besides the numbers, it also presents various functionalities such as, their biological activities, the

aromatic and palliative characteristics and the natural pigmentation, being one of the most diversified group of all (Kutchan et al., 2015c).

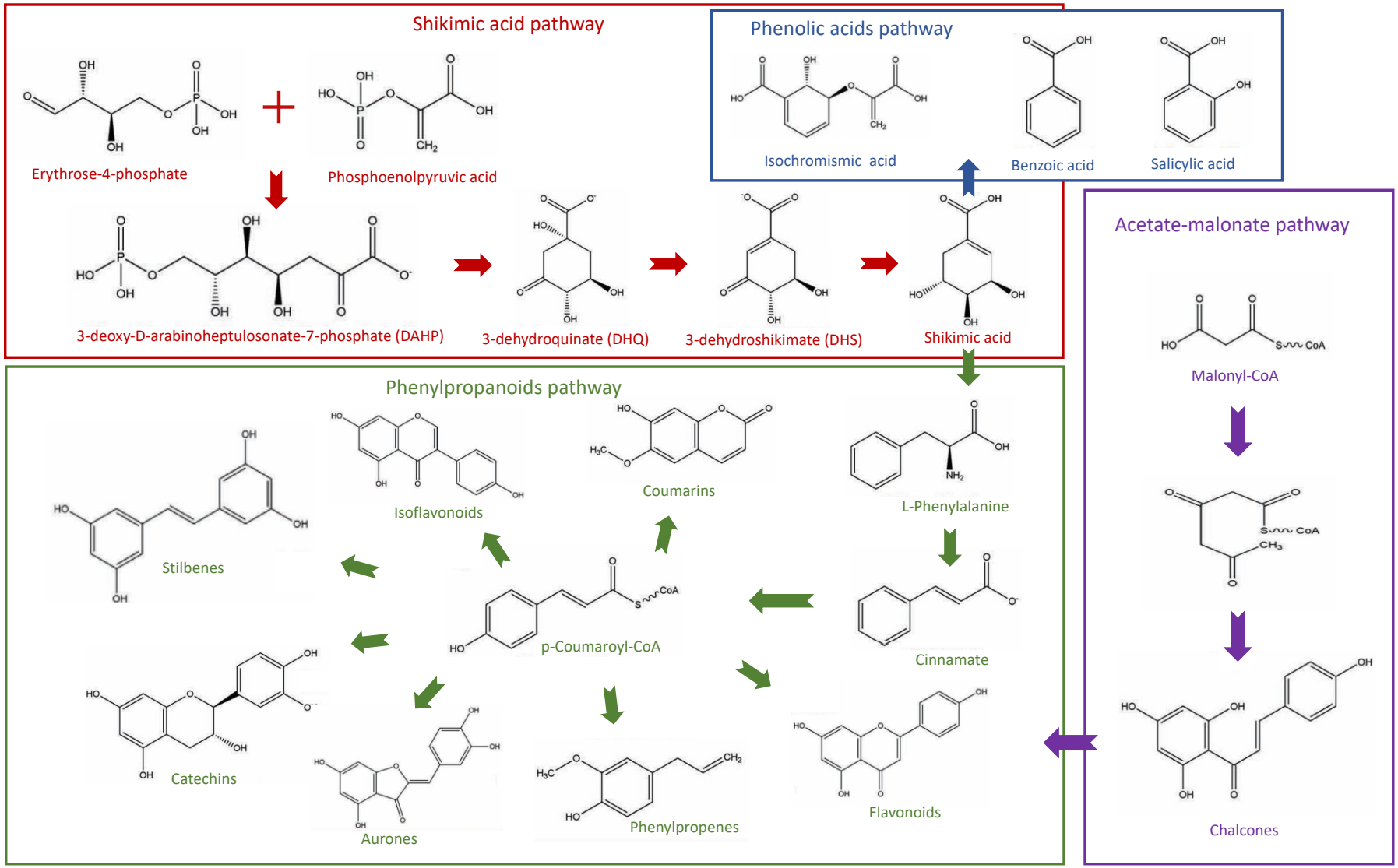


Figure 3. Metabolic pathways for the production of phenolic compounds (adapted from Babenko et al., 2019)

### 2.3.1. Roles in plants

Phenolic compounds have a significant importance in plants' growth, development and subsistence. Studies report that they were crucial in the times of life's transition to Earth´s crust in order to overcome the challenges of a terrestrial environment (Kutchan et al., 2015c). For example, flavonoids, a subclass of phenolic compounds, protect plants from the harmful UV radiations, by absorbing them; suberins, provided a hydrophobic barrier in the periderm of the roots and barks preventing water loss in desiccation times; lignin, help plants fighting gravity by giving more stability to the structure of cell walls in order to overcome their contestants (taller plants) for sunlight; stilbenes, coumarins, and furanocoumarins dissuade any kind of direct threat such as herbivores, insects or pathogens (virus, fungi and bacteria); and diarylheptanoids, gingerols and phenylphalenones helped the plants to proliferate by attracting pollinators by scents and colors . (Yang et al., 2016; Kutchan et al., 2015c; Babenko et al., 2019). Nevertheless, phenolics have less primal but also very important tasks, such as the regulation of auxin (hormones) transport, the prevention of oxidation of plant tissues and in the electrons transport chains of mitochondria and chloroplasts (Babenko et al., 2019).

### 2.3.2. Roles in humans

Phenolics just like terpenoids, possess a great number of applications, in human society. This diversification is generally given by the ability of the plants to provide that same variety to themselves, thus the more diverse are the substances roles in plants, the more they can be in human's industries. Therefore, phenolics are mostly used in food, supplementation and pharmaceutical industries. There are other applications, but with less relevance in today's world.

For the pharmaceutical industries, several examples can be given. Fortunately, a great number of phenolic compounds have showed strong antioxidant, anti-inflammatory, antihepatotoxic, anti-cancer, antiviral activities, etc. (Yang et al., 2016; Teoh, 2016; Puri, 1998b). Currently, some are already used in the treatment of the many illnesses or are being studied for future applications, as reported by Yang et al., (2016) and Teoh, (2016).

In a similar market, (i.e., the nutraceuticals market) phenolics-enriched food is also being used in the treatment/prevention of arthritis or diabetes, among many others (Lin et al., 2016), or just in fortification of general health given its various activities.

In terms of food applications, phenolics can be used as food pigments, flavours or even scents (example of vanillin, a simple phenol) to food products (Teoh, 2016). They can also be implemented in the preservation of food products given their antioxidant and antimicrobial activities in active packaging or even as additives (Martillanes et al., 2017).

# Chapter 3. Extraction Technologies in Plants

As mentioned earlier, plants are well known as an exceptional source of bioactive substances (Saito, 2010). Through history, they have been used for a wide variety of purposes (medicine, nutrition, clothing, biofuels, construction, etc.), but for an effective use in health and nutritional applications it is necessary to extract these functional compounds. Therefore, the selection of the most suitable extraction method has a tremendous importance in the quality and quantity of the products produced (Smith, 2003; Sasidharan et al., 2011). For such, several parameters have to be considered, including but not limited to, the chemical class of interest (flavonoids, polyphenols, alkaloids, oils, etc.) and the corresponding precautions (thermosensibility, photosensitivity, pH, etc.), the specific part of the plant to be extracted (leaves, flowers, bark, seeds, fruits, root, etc.), the matrix nature of the product (mixtures or pure substances), time availability, extraction yield, scale of extraction and financial resources (Belwal et al., 2017; Gupta et al., 2012). Methodologically speaking, there are many techniques available, from the most conventional to the most innovative technologies. Practically, it is very difficult to choose one single type of technique or method, as each one has its advantages and disadvantages. Thus, the most common and effective way to proceed is to adopt a combination of techniques (Azmir et al., 2013).

## 3.1. Conventional extraction techniques

Initially, the extraction of bioactive substances was performed by various classical extractions techniques. These methods have been used since ancient civilizations up until the 19$^{th}$ century with some modern updates through time (Azmir et al., 2013). The referred techniques are maceration, infusion, digestion, percolation, decoction, soxhlet and

hydrodistillation, each one with theirs advantages and disadvantages (Azmir et al., 2013; (Belwal et al., 2017). Most of them, are based in the extracting power of different solvents/solvent concentrations, and the application of mixing and/or heat to perform the extractions. In terms of efficiency, the choice of which solvent is used plays an extremely crucial role, where parameters such as polarity, solubility, concentration, pH and co-solvent have to be considered in order to obtain the targeted substances in a secure manner (Belwal et al., 2017).

Even with all of the precautions mentioned above, the conventional methods still present various disadvantages, for example, long extraction time, solvent toxicity, evaporation of large amount of solvent, the requirement of expensive and pure solvents, the thermal decomposition of sensitive compounds, and low extraction selectivity and yield (Belwal et al., 2017).

## 3.2. Non-conventional extraction techniques

With the growing demand for plants' bioactive substances, the discovery of new extraction methodologies received a renewed look. This interest emerged with the purpose of solving extraction limitations from old techniques, especially cost, yield and selectivity. In order to overcome these limitations several technologies were proposed, for instance, ultrasound extraction, enzyme extraction, microwave extraction, pulsed electric field extraction, supercritical fluid extraction and pressurized liquid extraction (Azmir et al., 2013; Belwal et al., 2017; Li et al., 2014). In the same way, just as with the older methods, solvents have to be carefully selected. However, the selection of the solvent does not depend solely on the targeted compounds, but also on the chosen extraction process (Belwal et al., 2017). To this end, rheological, thermophysical and parameters of other nature have to be considered, such as, solvent power, boiling point, reactivity, viscosity, recovery, vapor pressure, safety and cost (Belwal et al., 2017).

Aside from technical limitations, the environmental impact was also an important factor in the development of new extraction techniques. Currently, several agencies/organizations such as the European Environment Agency (EEA), the United States Environmental Protection Agency (EPA), the United Nations (UN) and others emphasise the necessity for this technological change on behalf of sustainability. Apart from some of the referred techniques (Azmir et al., 2013) being already in agreement with the standards of the Green Chemistry principles (Ivanković et al., 2017), the adaptation process is long from completed, and is of great importance that this mindset/framework endures for the next generations.

## 3.3. Purification processes

Since extractions diffuse every type of compounds to the solvent matrix at different rates, given the chosen affinity, the whole should be a very complex mixture of bioactive substances (Durazzo et al., 2018). Therefore, a purification process will be required in order to have a characterized product. The specificity of the characterization and identification will be defined by the producer itself, having in consideration the segmentation market in which it is inserted, pharmaceutical, nutritional, cosmetic or others. In other words, the final product can be presented as pure compounds, compound groups (phenolics, alkaloids, terpenoids, etc.) or unspecified mixtures.

Methodologically speaking, the techniques can be divided into two groups, the chromatographic techniques and the non-chromatographic techniques (Sasidharan et al., 2011).

### 3.3.1. Chromatographic techniques

The separation process of chromatography is based in the size, shape and charges of the molecules in question, and can be performed in the three different physical states (Sasidharan et al., 2011). Its adaptability is

one of its great advantages as can be seen by the great variety of methods that have been made over the time, such as the column, ion-exchange, gel-permeation (molecular sieve), affinity, paper, thin layer, gas, dye-ligand, hydrophobic interaction, pseudoaffinity and high-performance liquid chromatography (HPLC) (Coskun, 2016; Ingle et al., 2017). These methods have their own specifications (Coskun, 2016; Ingle et al., 2017), but the system principles are all based in the stationary and mobile phase, where the exchanges take place. This type of method is widely used in industry and in research institutes given its extremely high sensibility and separation effectiveness, being able to separate, identify and quantify almost every sort of compound (amino acids, proteins, nucleic acids, hydrocarbons, carbohydrates, drugs, etc.) (Coskun, 2016).

### 3.3.2. Non-chromatographic techniques

For the non-chromatographic techniques, there are not many possibilities to quantify compounds. Most of the techniques are more directed to the fields of research where the characterization and identification of substances are more relevant. In this view, techniques such as Fourier-transform infrared spectroscopy (FTIR), Nuclear magnetic resonance spectroscopy (NMR), Mass spectrometry (MS) and Phytochemical screening assay have a great importance, each one with its own strengths (Sasidharan et al., 2011; Ingle et al., 2017).

Nevertheless, there are alternatives such as the immunoassays, which uses monoclonal antibodies, to immobilize drugs and low molecular weight natural bioactive compounds (Sasidharan et al., 2011). This type of method is not wide-ranging, but in some cases these methods are more sensitive than the conventional HPLC methods, thus proving to be valuable tools (Sasidharan et al., 2011).

# Chapter 4. Methodologies to Determine Biological Activity

With a proper preparation, bioactive compounds can provide several benefits to health. These benefits are given through the chemical interactions that an active ingredient provides to living organisms, being acknowledged as biological activity. However, it is of great importance to monitor all of these chemical interactions since the first activity of a substance is its toxicity, in relation to the dosage (Dwyer et al., 2018). In this regard, several examples showed that substances of high toxicity to humans, can offer unthinkable benefits in the right dosage and lead to death in the wrong dosage, as was the case with some alkaloids mentioned before (e.g., cocaine, morphine, nicotine, etc.) (Kutchan et al., 2015b). Nevertheless, with the proper knowledge most dangerous substances can be leveraged for a greater good.

In terms of functionality, just like the diversity of the phytochemical world, the biological activities are equally vast taking as examples the antioxidant, anti-inflammatory, antibacterial, antifungal, anticancer, hepatoprotective, immunomodulatory, cardiovascular protective, antispasmodic, sedative, anticoagulant, antiviral, diaphoretic activities, etc. (Barnes et al., 2007).

## 4.1. Antioxidant Activity

The core purpose of antioxidants is clearly explicit within its terminology: to prevent oxidation reactions. These reactions take place because, there is a transfer of hydrogen or electrons from substances to an oxidizing agent, which can be any kind of substance ready to receive these charges (Moharram, 2014). In the absence of an antioxidant agent,

oxidation reactions will produce free radicals (Moharram, 2014). These radicals represent a serious threat, since they can start chains reactions that were not supposed to happen, which may cause damage to cells or even lead to their death (Moharram, 2014). Besides the interference that it has in organisms, they also have a significative impact in the deterioration of food products, putting at risk the quality and safety of the products (Shahidi, 2015).

In order words, antioxidant agents prevent these chain reactions by removing free radicals as intermediaries of oxidation. However, there are two forms of free radicals being replaced. The first is when the antioxidants act as a competing substrate for the reaction (oxidising themselves) with the free radicals, whereas the second form is when the antioxidant agent directly stabilises the radical preventing it from intervening in other reactions (Moharram, 2014). Although, this last form is not considered an antioxidant activity, but rather an antiradical activity being sometimes difficult to define after all which activity is the compound in question generating, especially in methods that use stable free radicals (DPPH, ABTS, etc.) (Moharram, 2014).

### 4.1.1. Types of oxidation reactions

Free radicals are the product of a normal metabolism of an organism. They are associated to several processes in the human body, such as inflammation processes, phagocytosis, physical exercises, mitochondria through xanthine oxidase and many others (Lobo et al., 2010). However, external factors can also contribute to the production of free radicals, for example: environmental pollutants, radiation, smoking, drugs, ozone, industrial solvents and pesticides (Lobo et al., 2010). Therefore, antioxidants help to control this dispute and balance the quantity of free radicals, although not always this balance can be normally stabilize leading the organism to an oxidative stress (Carocho and Ferreira, 2013). This stress can be segmented in at least four types of targeted oxidations, for

example the proteins' oxidation, the DNA and RNA' oxidation, sugars' oxidation and lipids' oxidation (the most problematic type of oxidation in the food industry field) (Carocho and Ferreira, 2013). For a better understanding of this subject, Carocho and Ferreira (2013) address several specifications that each type of oxidation reaction may have.

### 4.1.2. Types of antioxidants

In this categorization, there are only two types of antioxidants, the enzymatic and the non-enzymatic ones (Moharram, 2014).

Within the enzymatic antioxidants are the primary and the secondary (Moharram, 2014). The primary enzymes (superoxide dismutase, catalase and glutathione peroxidase) prevent the formation or neutralize free radicals, while the secondary enzymes (glutathione reductase and glucoase-6-phosphate-dehydrogenase) do not neutralize directly the radicals but have supporting roles to other antioxidants (Carocho and Ferreira, 2013). For instance, the enzyme, glucose-6-phosphate-dehydrogenase regenerates NADPH (nicotinamide adenine dinucleotide phosphate), and this process creates a reducing environment, which supports the action of other endogenous antioxidants (Carocho and Ferreira, 2013).

For the non-enzymatic antioxidants, in a general idea, most of them can be acquired from dietary sources (Moharram, 2014). There is a great variety of substances with this functionality, with a special focus in phenolics (flavonoids, carotenoids and phenolic acids) presenting itself as the largest class (Carocho and Ferreira, 2013). Nevertheless, other classes provide antioxidant activity such as, vitamins and derivatives, cofactors, minerals, organosulfur compounds and nitrogen non-protein compounds (Carocho and Ferreira, 2013).

### 4.1.3. Determination methods

For the screening/test of antioxidant activity, the literature is quite extensive, especially given the very different oxidation reactions that can occur, as mentioned earlier. In Table 3 shows some of the most relevant tests, but the available methodologies are not limited to those presented. Depending on the focus of the study, there are also other methods that allow, for example, the quantification of radicals' presence (Antolovich et al., 2000; Gutteridge, 1995).

Table 3. List of the most important assays to screen antioxidant activity

| Assay | Mechanism | Reference |
|---|---|---|
| ABTS (2,20-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) | Scavenging activity | Antolovich et al. (2000) |
| DPPH (2,2-diphenyl-1-picrylhydrazyl) | Scavenging activity | Antolovich et al. (2000) |
| $HO^-$ scavenging activity | Scavenging activity | Huang et al. (2005) |
| $H_2O_2$ scavenging activity | Scavenging activity | Huang et al. (2005) |
| $O_2^-$ scavenging activity | Scavenging activity | Huang et al. (2005) |
| Peroxynitrite (ONOO) scavenging capacity | Scavenging activity | Huang et al. (2005) |
| FRAP (ferric reducing antioxidant power) | Reducing power | Antolovich et al. (2000) |
| Conjugated diene | Lipid peroxidation inhibition | Moon and Shibamoto (2009) |
| FOX (ferrous oxidation-xylenol) | Lipid peroxidation inhibition | Moon and Shibamoto (2009) |
| FTC (ferric thiocyanate) | Lipid peroxidation inhibition | Moon and Shibamoto (2009 |
| GSHPx (glutathione peroxidase) | Lipid peroxidation inhibition | Gutteridge (1995) |
| Heme degradation of peroxides | Lipid peroxidation inhibition | Gutteridge (1995) |
| Iodine liberation | Lipid peroxidation inhibition | Gutteridge (1995) |
| TBARS (thiobarbituric reactive substances) | Lipid peroxidation inhibition | Gutteridge (1995) |
| TEAC assay (Trolox equiv. antioxidant capacity) | Antioxidant activity | Huang et al. (2005) |
| Total oxidant potential using Cu (II) as an oxidant | Antioxidant activity | Huang et al. (2005) |
| TRAP (total radical-trapping antioxidant parameter) | Antioxidant activity | Antolovich et al. (2000) |
| ACA (aldehyde/carboxylic acid) | Slow oxidation phenomena | Moon and Shibamoto (2009 |

# Part II – Experimental Work

# Chapter 5. Materials and Methods

## 5.1. Extracts' Production

### 5.1.1. Solvents' preparation

Three extractants were used in this procedure: a mixture of ethanol/water (40:60, w/w), a mixture of glycerol/water (75:15, w/w) and ethanol of 96 % (v/v). To this purpose, in the first case 400 g of ethanol (96 % (v/v)) were added to 600 g of distilled water and in for the second extractant 1260 g of glycerol (99.5 %) were added to 420 g of distilled water and finally mixed. For the last solvent, it was not necessary any preparation since it already was bought at the necessary concentration.

### 5.1.2. Herbs' preparation

The herbal substances were acquired from a Czech distributor (bylik.cz), that already pre-treated the plants. They were already dried and grinded except for rose hip fruit (No. 5) and elderberry fruit (No. 8) that required an additional crushing and grinding with the mortar. Consequently, was weighed (Kern 440 Laboratory Balance) 3 g of each herb into small vials.

### 5.1.3. Extraction stage

The extraction method used was maceration (extraction at room temperature for a stipulated duration, with occasional mixing at every 2/3 days). Every herb had three different vials, each for one of the solvents. Each of them was filled with the relevant solvent up to the optimal minimal

ratio, having in consideration the absorption rate of each one. After the fifth day, all were corrected to a ratio of 1 g of herb to 10 g of solvent (1:10 (w/w)). In total the extraction process was done for 14 days.

Being the absorption process so different from each plant, some had to be adjusted beyond the ratio mark in order soak all the biomass. By that reason, the following herbs were adjusted into the respective ratios: red clover (No.6) to 1:17, wild strawberry (No.13) to 1:11, common hop (No.17) to 1:20 and ramson/wild garlic (No.31) to 1:15 in the 40 % ethanol extracts (E40); red clover (No.6) to 1:15 and common hop (No.17) to 1:17.5 in the 96% ethanol extracts (E96). For glycerol extracts (G75), the process was similar, but the ratios were scaled to 1:15, 1:20 and 1:25, depending on the herb.

### 5.1.4. Purification process

In order to obtain a clear extract, each herb was manually pressed through a nylon net and a paper sheet to maximize the physical extraction and then through a vacuum filtration (KNF LABOPORT N 820 vacuum pump and paper filters of 90 mm in diameter were used) to separate the smaller particles within the extract. The extracts were then stored in small vials. For biological analysis, approximately 6 mL of each extract was centrifuged (Roth micro centrifuge SD) and stored in other small vials

## 5.2. Dry weight measurement

### 5.2.1. Solvent evaporation

From each herb and each solvent (only the alcoholics extracts), 10 mL of herb extract was pipetted into glass tubes and then placed in a food (Klarstein Food Oven) oven at 3 different temperatures (40 °C for 5 days, 55 °C for the next 2 days and 70 °C for the last 8 days).

### 5.2.2. Determination of dry weight

To the determination of the dry weight, the glass test tubes were weighted (Kern ADJ 100-4 Analytic Balance) before having the extracts ($W_1$) and after ($W_2$) the evaporation.

Equation 1 was used in order to calculate the bioactive compounds mass (*mBC*) (in mg) per mass (g) of herb, where the Ratio (*R*) is defined by the mass of herb per mass of solvent, *V* is the sample volume, $\rho$ is the density of solvents.

$$mBC\ /\ (\mathrm{mg})/\mathrm{g} = [(\frac{\frac{W_2 - W_1}{V}}{\rho}) \times R^{-1} \times 1000] \quad \text{Equation 1}$$

## 5.3. Antioxidant Activity Measurement

### 5.3.1. Solutions' preparation

A solution of DPPH (2,2-diphenyl-1-picrylthydrazyl (99 %)) was prepared with a concentration of 0.5 g/L, therefore 0.1 g of DPPH was dissolved in 200 mL of DMSO (Dimethyl sulfoxide (99.5 %)). Given its thermic a visual radiation sensibility, the solution was stored with aluminum foil in the cold.

A solution of vitamin C with a concentration of 1 g/L was also prepared, then 0.1 g of ascorbic acid were dissolved into 100 mL of distilled water. For the preparations of subsequent solutions of vitamin C, dilutions of this main stock solution were used.

### 5.3.2. Calibration curve

Initially, a calibration curve (Appendix A) was created with 9 solutions of vitamin C (ascorbic acid (99 %)) in different concentrations (0.1-0.4 g/L) to cover the linear zone of the regression, which were read in an optical density reader ((ODR) – Bioscreen C MBR) at room temperature on a

wavelength of 540 nm. The experimental assay was performed by pipetting 25 μL of vitamin C solution, 50 μL of distilled water and 75 μL of DPPH into a well. This was repeated for other concentrations in quadruplicate, and it was read with an ODR in the beginning and the end, 6 hours later.

### 5.3.3. Herbal testing

The antioxidant activity test of the herb extracts was proceeded in the same way as the calibration curve, but instead of the vitamin solutions it was used the extracts, with vitamin C as positive control, on a concentration of 0.5 g/L, and a negative control with just distilled water.

### 5.3.4. Percentage of antioxidant's inhibition

The determination of the antioxidant inhibition (*AI* in percentage) was proceeded by the difference between the absorbency of the negative control·*NC* (absence of any type of inhibitor) and the absorbency of the sample (*SA*) in question (Equation 2).

$$AI = \frac{NC - SA}{NC} \times 100 \qquad \text{Equation 2}$$

### 5.3.5. Vitamin C equivalents

Vitamin C is well known by its powerful antioxidant activity, and by its extensive use in the supplements' market. For that reason, vitamin c is an excellent reference for uncharacterised extracts. For the determination of vitamin C equivalents (*c*), the calibration curve previously prepared (Appendix A) was used (Equation 3).

$$c \text{ eq. Vit. C}/(mg/L) = \frac{SA - 2.328}{-4.4195} \times 1000 \qquad \text{Equation 3}$$

## 5.4. Flavonoids' Quantification

### 5.4.1. Solutions' preparation

For this measurement, four solutions were prepared: aluminum chloride ($AlCl_3$), sodium nitrate ($NaNO_2$), sodium hydroxide ($NaOH$) and ethanol 30 %. Aluminium chloride ($AlCl_3$ 10 % v/v)) solution was prepared by weighting 4.8 g of $AlCl_3$, and 17.95 g of distilled water into a flask, under agitation; sodium nitrate ($NaNO_2$ 5 % (v/v)) solution it was weight 5.43 g of $NaNO_2$and 47.36 g of distilled water into a flask, under agitation; sodium hydroxide ($NaOH$ 1mol/L) solution was prepared by adding, 4 g of $NaOH$ to 100 mL of distilled water, under agitation; ethanol 30 % (v/v) was prepared by mixing 31.25 mL of ethanol 96 % (v/v) with 68.75 mL of distilled water.

A solution of rutin with a concentration of 5 g/L was also prepared: 0.05 g of rutin (94 %) were dissolved into 10 mL of DMSO. For the preparations of subsequent solutions of rutin, dilutions of this main stock solution were used. All of these solutions were stored under refrigeration.

### 5.4.2. Calibration curve

Firstly, a calibration curve of flavonoids equivalents (Appendix B) was prepared with 18 solutions of rutin at different concentrations (0-5 g/L).

The experimental assay was performed by pipetting 20 μL of rutin solution, 14 μL of $NaNO_2$, and 200 μL of ethanol 30 % into a well (96-well microplate), and this mixture was agitated and let to rest. After 6 min, were added 14 μL of $AlCl_3$, and again let to rest another 5 min. At last, 100 μL of $NaOH$ and 152 μL of ethanol 30 % were added and agitated in the wells. After another period of rest (40 min), the microplate was read in an ODR (Bioscreen C MBR) at 420 nm

### 5.4.3. Herbal testing

The flavonoids' quantification of the herb extracts was proceeded in the same way as the calibration curve, but instead of the rutin solutions it was used the extracts, with rutin as positive control, on a concentration of 0.5 g/L, and a negative control with just distilled water.

### 5.4.4. Flavonoids' equivalents

For the determination of flavonoids' equivalents concentration (*C*), the calibration curve previously prepared (Appendix B), was used (Equation 4).

$$c\ \text{Flav. eq.}/(mg/L) = \frac{SA - 0.107}{0.000492} \qquad \text{Equation 4}$$

### 5.4.5. Flavonoids' percentage in the biocompounds extracts

Equation 5 was used for the determination of flavonoids' percentage (*FP*)in the total mass of bioactive compounds (*mBC*).

$$FP = \left(\frac{\frac{C\ Flav.eq.}{\rho} \times R^{-1}}{mBC}\right) \times 100 \qquad \text{Equation 5}$$

## 5.5. Sensory Analyses

Organoleptically, only the taste and flavour were tested, within a scale of 0-10, from the worst to the best. All extracts were tested, but in two of the solvents, it was needed some dilution given the alcoholic strength of the herbal extracts. The test was performed as an informal degustation.

## 5.6. Optimization process

The optimization process was designed using the software Design Expert (Version 11.1.0.1), with the purpose of finding the extraction conditions where the extraction of flavonoids would be maximized. The type of study chosen was Response Surface and the design type was Central composite with four input variables (temperature, solvent ratio, time of extraction and solvent concentration) and one output variable (flavonoids' concentration). Tables 4 and 5 present the extraction conditions of the plants, common hop and sea-buckthorn, for the experiment.

The technical procedures, regarding the solvents' preparation, extraction process, extract's purification and flavonoids' quantification were performed in the same way as mentioned earlier.

Following the experimental procedures, the collected data was then inserted in the program and through mathematical models, the optimal conditions were calculated.

Table 4. Extraction's conditions for the optimization process of common hop

| Run | Temperature/(°C) | Ratio/(w/w) | Time/(days) | % EtOH (w/w) |
|---|---|---|---|---|
| **1** | 22 | 9 | 10 | 70 |
| **2** | 40 | 6 | 1 | 96 |
| **3** | 5 | 6 | 10 | 40 |
| **4** | 40 | 6 | 10 | 96 |
| **5** | 40 | 9 | 6 | 70 |
| **6** | 22 | 9 | 6 | 96 |
| **7** | 40 | 12 | 10 | 40 |
| **8** | 22 | 6 | 6 | 70 |
| **9** | 22 | 9 | 6 | 70 |
| **10** | 22 | 9 | 6 | 70 |
| **11** | 22 | 9 | 1 | 70 |
| **12** | 40 | 12 | 1 | 40 |
| **13** | 22 | 12 | 6 | 70 |
| **14** | 5 | 9 | 6 | 70 |
| **15** | 5 | 12 | 1 | 96 |
| **16** | 22 | 9 | 6 | 70 |
| **17** | 5 | 12 | 10 | 96 |
| **18** | 22 | 9 | 6 | 70 |
| **19** | 22 | 9 | 6 | 40 |
| **20** | 5 | 6 | 1 | 40 |

Table 5. Extraction's conditions for the optimization process of sea-buckthorn

| Run | Temperature/(ºC) | Ratio/(w/w) | Time/(days) | % EtOH (w/w) |
|---|---|---|---|---|
| **1** | 22 | 4.5 | 10 | 70 |
| **2** | 40 | 3 | 1 | 96 |
| **3** | 5 | 3 | 10 | 40 |
| **4** | 40 | 3 | 10 | 96 |
| **5** | 40 | 4.5 | 6 | 70 |
| **6** | 22 | 4.5 | 6 | 96 |
| **7** | 40 | 6 | 10 | 40 |
| **8** | 22 | 3 | 6 | 70 |
| **9** | 22 | 4.5 | 6 | 70 |
| **10** | 22 | 4.5 | 6 | 70 |
| **11** | 22 | 4.5 | 1 | 70 |
| **12** | 40 | 6 | 1 | 40 |
| **13** | 22 | 6 | 6 | 70 |
| **14** | 5 | 4.5 | 6 | 70 |
| **15** | 5 | 6 | 1 | 96 |
| **16** | 22 | 4.5 | 6 | 70 |
| **17** | 5 | 6 | 10 | 96 |
| **18** | 22 | 4.5 | 6 | 70 |
| **19** | 22 | 4.5 | 6 | 40 |
| **20** | 5 | 3 | 1 | 40 |

## 5.7. Statistical analyses

Experiments were performed at least in triplicate, and in some cases more. The mean values and standard mediations of those determinations were calculated and presented.

For the presented regressions, the statistical analysis was conducted by the software Microsoft Excel for a 95 % confidence interval.

For last, the statistical data presented in the optimization process was presented and calculated by the program Design Expert (Version 11.1.0.1)

# Chapter 6. Results and Discussion

## 6.1. Extraction ratios' study

Previously, during the preparation of the extraction process (maceration), a survey on the extraction ratios was performed in order to acknowledge which were the most suitable ratios for each herb under study. The analysis was made in all 3 solvents and the results are presented in Table 6.

This research has the purpose of finding what would be the minimal optimal ratio for extraction for each herb, taking into consideration the absorption rate of each plant for each of the solvents. The minimal optimal ratio in this situation, is defined as the minimal quantity of extractant that soaks all the biomass (submersion of the biomass). The submersion is a requirement, in order to facilitate the diffusion of bioactive compounds to the solvent matrix.

As shown in Table 6, the most alcoholic solvent (96 % (v/v) of ethanol) is in general, the extractor that needs lower amounts of solvent to soak all the biomass. In the other hand, 75 % (w/w) of glycerol is the extractant that requires the highest quantity of solvent to soak the biomass. These differences in quantity are derived from physical parameters such as viscosity, solubility and polarity that promote or prevent the permeation of the extractant in the biomass (Abubakar and Haque, 2020).

Therefore, this information will help in the forthcoming work, by preventing the economic and environmental waste of solvents in scale-up projects.

Table 6. Optimal minimal ratios of each herb in the three different solvents

| No. | Plant Name | Optimal minimal ratio (E40) | Optimal minimal ratio (E96) | Optimal minimal ratio (G75) |
|---|---|---|---|---|
| 1 | Dried burdock | 1: 3.5 | 1:1.5 | 1:5 |
| 2 | Couch grass | 1: 4 | 1: [2-2.5] | 1:4.5 |
| 3 | Silver birch | 1: [9-9.5] | 1: [7-7.5] | 1:15 |
| 4 | Sea-buckthorn | 1:3 | 1:2 | 1: [2.5-3] |
| 5 | Rose hip | 1:2 | 1:1.5 | 1: [2.5-3] |
| 6 | Red clover | 1:17 | 1:13 | 1:21 |
| 7 | Common mallow | 1: [6-6.5] | 1:4.5 | 1:12 |
| 8 | Elderberry | 1: [2-2.5] | 1:1.5 | 1: [3-3.5] |
| 9 | Galega/ Goat's-rue | 1: [8-8.5] | 1:5.5 | 1:13 |
| 10 | European Goldenrod | 1:6 | 1:4 | 1:10 |
| 11 | Hawthorn | 1:6 | 1:4.5 | 1:12 |
| 12 | Yarrow | 1: [7-7.5] | 1:5.5 | 1:12 |
| 13 | Wild Strawberry | 1: [11-11.5] | 1:8.5 | 1:15.5 |
| 14 | Heather | 1: [6.5-7] | 1:4 | 1:12 |
| 15 | Dandelion | 1:3 | 1:1.5 | 1:5 |
| 16 | Common Soapwort | 1: [3.5-4] | 1: [1.5-2] | 1:5.5 |
| 17 | Common hop | 1:20 | 1:17.5 | 1:27 |
| 18 | Garlic | 1: [3.5-4] | 1:1.5 | 1:5.5 |
| 19 | Small-leaved lime | 1: [7-7.5] | 1:4 | 1:12 |
| 20 | Yellow sweet clover | 1: [6.5-7] | 1:4.5 | 1:9.5 |
| 21 | Wormwood | 1:5.5 | 1:3.5 | 1:10 |
| 22 | St John's-wort | 1:6 | 1:4 | 1:10 |
| 23 | Dandelion | 1: [7-7.5] | 1:6.5 | 1:13 |
| 24 | Motherwort | 1:9.5 | 1:6 | 1:13.5 |
| 25 | Common comfrey | 1:4.5 | 1:1.5 | 1:5.5 |
| 26 | Daisy | 1:8 | 1:6.5 | 1:14 |

Table 6. Optimal minimal ratios of each herb in the three different solvents (continuation)

| No. | Plant Name | Optimal minimal ratio (E40) | Optimal minimal ratio (E96) | Optimal minimal ratio (G75) |
|---|---|---|---|---|
| 27 | Lingonberry | 1:5.5 | 1:4 | 1:7 |
| 28 | Urtica | 1:7.5 | 1:5.5 | 1:12 |
| 29 | Hemp nettle | 1: [9-9.5] | 1:5.5 | 1:15 |
| 30 | Basil | 1:8 | 1:5.5 | 1:12 |
| 31 | Ramson, Wild garlic | 1:15 | 1: [8-8.5] | 1:20 |
| 32 | Veronica | 1: [9-9.5] | 1:7 | 1:14 |
| 33 | Ground-ivy | 1: [8.5-9] | 1:6.5 | 1:14 |
| 34 | Common Agrimony | 1:7.5 | 1:4.5 | 1:11 |
| 35 | Valerian | 1: [3-3.5] | 1: [2-2.5] | 1:3.5 |
| 36 | White horehound | 1: [8.5-9] | 1:5.5 | 1:11 |
| 37 | Dyer's greenweed | 1: [5.5-6] | 1:4 | 1:10 |
| 38 | Liquorice | 1: [4.5-5] | 1:3 | 1:6.5 |
| 39 | Horsetail | 1:10 | 1:6.5 | 1:15 |
| 40 | Buckwheat | 1: [9.5-10] | 1: [5.5-6] | 1:12.5 |
| 41 | Calendula | 1:9.5 | 1:8 | 1:15.5 |
| 42 | Sage | 1:7.5 | 1:5 | 1: [10-10.5] |
| 43 | Breckland thyme | 1:8.5 | 1:5 | 1:11.5 |
| 44 | Oregano | 1: [6.5-7] | 1: [4-4.5] | 1:10.5 |
| 45 | Willow | 1:5 | 1:3 | 1:6 |
| 46 | Rosemary | 1:5.5 | 1:3.5 | 1:7 |
| 47 | Chamomile | 1:10 | 1: [7-7.5] | 1:15.5 |
| 48 | Elder flower | 1:6 | 1:3.5 | 1:12.5 |
| 49 | Wall germander | 1:6.5 | 1:4.5 | 1:10 |
| 50 | White Mustard | 1:1.9 | 1:1 | 1:2.5 |
| 51 | Common chicory | 1:5 | 1:2.5 | 1:7.5 |
| 52 | Milk thistle | 1:2 | 1: [1-1.5] | 1:2.5 |

## 6.2. Organoleptic testing

Being this work directed to food industry, more specifically to the supplements' market, the taste and flavour of the products (alcoholic and non-alcoholic extracts) play an important role in market acceptance by the consumers.

In this regard, an organoleptic test was conducted, testing only the taste and flavour, rating just the basic tastes (sweet, sour, salty, bitter and umami) in a scale of 0-10, being 0 the worst and 10 the best. The methodology of this test was quite easy, the extracts were simply tasted from the crude extract, exceptionally the most alcoholic extracts (96 % of ethanol) that were diluted in a portion of 1/3, given the extremely high volume of alcohol. The results of the sensory tests are then presented in Table 7.

As it is perceptible by the data in the Tables 7, the most alcoholic extracts are the most unpleasant ones, experience emphasized from the high volume of alcohol in the extracts that highlights the sour and bitter notes in a distasteful way. The glycerol extracts, on the other hand, are the most enjoyable, being in general sweet, highlighting in some cases the floral and herbal flavours.

There are some herbs that are frequently in the top five in different solvents, such as silver birch, common hop and chamomile. As the extracts are usually sold in mixes of several plants, besides the bioactive functionality these herbs, also provide an organoleptic action improving the tasting experience, being as well as a viable option in the marketplace.

This option of extracting bioactive compounds with alcoholic and non-alcoholic solvents had the purpose of extending the market window, thus selling the alcoholic extracts to adults and the glycerol extracts to children since these extracts will provide a more pleasant tasting experience.

Table 7. Organoleptic test results of the herb extracts of three different solvents

| No. | Plant Name | Taste and flavour E40* | Taste and flavour E96* | Taste and flavour G75* |
|---|---|---|---|---|
| 1 | Dried burdock | Bitter (5.5)/5 | Bitter (2.5)/2.5 | Sweet (5)/5 |
| 2 | Couch grass | Bitter (5)/4.5 | Bitter (4)/4 | Sweet (6.5)/6 |
| 3 | Silver birch | Sour (5.5)/5.5 | Sour (5.5)/5.5 | Sweet (7)/8 |
| 4 | Sea-buckthorn | Sour/Sweet (4.5)/4.5 | Sour/Sweet (4)/5 | Sweet (6.5)/6 |
| 5 | Rose hip | Sour/Sweet (5)/4.5 | Sour (4)/4 | Sweet (7)/7 |
| 6 | Red clover | Sour/Sweet (4.5)/5.5 | Sour/Sweet (4.5)/5 | Sweet (7)/6.5 |
| 7 | Common mallow | Sour/Bitter (5.5)/5.5 | Sour/Bitter (4)/4.5 | Sweet (7)/6 |
| 8 | Elderberry | Bitter (4.5)/5.5 | Bitter (3)/4 | Sweet (6.5)/5.5 |
| 9 | Galega/ Goat's-rue | Sour (5)/5 | Sour (6)/3.5 | Sweet (7)/5.5 |
| 10 | European Goldenrod | Bitter (4.5)/3.5 | Bitter (4)/3.5 | Sweet (7)/6.5 |
| 11 | Hawthorn | Bitter (6)/5.5 | Bitter (4.5)/5.5 | Sweet (6.5)/5.5 |
| 12 | Yarrow | Sour (4)/4 | Sour (4.5)/5 | Sweet (7)/6 |
| 13 | Wild Strawberry | Bitter (5)/5 | Bitter (5)/5.5 | Sweet (7)/7.5 |
| 14 | Heather | Bitter (4)/3.5 | Bitter (4)/3.5 | Sweet (7)/7.5 |
| 15 | Dandelion | Bitter (4)/3.5 | Bitter (2.5)/3.5 | Sweet (6.5)/5.5 |
| 16 | Common Soapwort | Sweet (4)/3.5 | Sweet (4)/5 | Sweet (6)/4 |
| 17 | Common hop | Sour/Sweet (4.5)/6 | Sour/Sweet (4)/6 | Sweet (7)/8 |
| 18 | Garlic | Bitter (5)/5.5 | Bitter (4.5)/4.5 | Sweet (6)/4 |
| 19 | Small-leaved lime | Sour (5)/6.5 | Sour (5)/6.5 | Sweet (7)/6 |
| 20 | Yellow sweet clover | Bitter (5)/7 | Bitter (3.5)/5.5 | Sweet (7)/6.5 |
| 21 | Wormwood | Sour (1)/1 | Sour (1)/1 | Sweet (4.5)/2.5 |
| 22 | St John's-wort | Sour (4)/4 | Sour (3)/3 | Sweet (7)/7 |
| 23 | Dandelion | Sour (4.5/4) | Sour (3)/4 | Sweet (7)/6 |
| 24 | Motherwort | Bitter (4)/4 | Bitter (2.5)/2.5 | Sweet (7)/6 |
| 25 | Common comfrey | Bitter (4)/3 | Bitter (1.5)/1.5 | Sweet (7)/5 |
| 26 | Daisy | Bitter (3.5)/3.5 | Bitter (3)/3 | Sweet (7)/7 |

*At green are the top five herbs in terms of taste and flavour.

Table 7. Organoleptic test results of the herb extracts of three different solvents (continuation)

| No. | Plant Name | Taste and flavour E40* | Taste and flavour E96* | Taste and flavour G75* |
|---|---|---|---|---|
| 27 | Lingonberry | Sour (3.5)/3 | Sour (3)/3 | Sweet (7)/6.5 |
| 28 | Urtica | Bitter (4)/4 | Bitter (4)/4.5 | Sweet (7)/5.5 |
| 29 | Hemp nettle | Bitter (4.5)/4 | Bitter (3.5)/3.5 | Sweet (7)/8 |
| 30 | Basil | Sour (4.5)/5 | Sour (4.5)/5 | Sweet (7)/5.5 |
| 31 | Ramson, Wild garlic | Bitter (5)/6.5 | Bitter (4.5)/5.5 | Sweet (7)/4.5 |
| 32 | Veronica | Bitter (4.5)/4 | Bitter (4)/4 | Sweet (7)/7 |
| 33 | Ground-ivy | Bitter (4.5)/4.5 | Bitter (5)/5 | Sweet (6.5)/5.5 |
| 34 | Common Agrimony | Bitter (4)/4 | Bitter (3.5)/4 | Sweet (7)/6.5 |
| 35 | Valerian | Bitter (5.5)/6.5 | Bitter (5)/3.5 | Sweet (7)/4.5 |
| 36 | White horehound | Sour (4)/2.5 | Sour (2)/2.5 | Sweet (7)/6 |
| 37 | Dyer's greenweed | Bitter (5)/5.5 | Bitter (4)/4.5 | Sweet (7)/7.5 |
| 38 | Liquorice | Bitter/Sweet (6)/8 | Bitter (2)/2 | Sweet (7)/5 |
| 39 | Horsetail | Bitter/Sweet (5)/6 | Bitter (4)/5 | Sweet (7)/5 |
| 40 | Buckwheat | Bitter (5)/5.5 | Bitter (5)/5 | Sweet (7)/5.5 |
| 41 | Calendula | Sour (4)/3 | Sour (1.5)/1.5 | Sweet (7)/5.5 |
| 42 | Sage | Sour (5)/5 | Sour (2)/3 | Sweet (7)/4.5 |
| 43 | Breckland thyme | Sour (6)/6 | Sour (5)/5.5 | Sweet (7)/6 |
| 44 | Oregano | Bitter (5)/5.5 | Bitter (3.5)/4 | Sweet (7)/6.5 |
| 45 | Willow | Bitter (4.5)/6 | Bitter (3.5)/2 | Sweet (7)/5.5 |
| 46 | Rosemary | Sour (4.5)/6 | Sour (3.5)/4 | Sweet (7)/5.5 |
| 47 | Chamomile | Sour (7)/9 | Sour (4.5)/6 | Sweet (7)/9.5 |
| 48 | Elder flower | Sour/Sweet (5.5)/6.5 | Sour/Sweet (4)/5 | Sweet (7)/5.5 |
| 49 | Wall germander | Bitter (5)/6 | Bitter (4)/3 | Sweet (7)/6.5 |
| 50 | White Mustard | Bitter (4)/4.5 | Bitter (2.5)/2.5 | Sweet (7)/5 |
| 51 | Common chicory | Bitter (3)/2.5 | Bitter (2)/2 | Sweet (7)/4.5 |
| 52 | Milk thistle | Bitter (4)/4.5 | Bitter (3.5)/3.5 | Sweet (7)/6 |

*At green are the top five herbs in terms of taste and flavour.

## 6.3. Antioxidant activity test

For testing the antioxidant potential, a chromatographic method was selected, in this case the DPPH method, where the radical DPPH is degraded into pale yellow or colourless, in case of having antioxidant agents, or stays dark violet if no reaction takes place. This method was chosen given its easiness and effectiveness in screening the antioxidant activity of the extracts. The scavenging activity results of the herbal extracts can be seen in Table 8 (the results can be seen in graphs in Appendix C).

The table present shaded in green the top five herbs and in yellow the next five herbs of the top ten, in terms of inhibition potential, for each solvent. The best results, in general, were obtained in the 40 % EtOH and 75 % glycerol extracts, with special attention to the herbs: silver birch, sea-buckthorn, rose hip, St John's wort and common hop that stayed in the top five in both solvents with the highest values of inhibition of the experiment (71-84 %), with the exception of common hop that was in the top five in the three solvents.

The inhibition results showed that the antioxidant potential peaked in different extraction solvents, on various herbal extracts. This occurrence is likely due to the fact that these different solvents/concentrations promote the diffusion of various compounds, some with more or less antioxidant capacity. There are several herbal examples where the best inhibition results are obtained in the 96 % EtOH extracts (common hop, heather, common comfrey, etc.), a solvent concentration, in terms of alcoholic concentration, less prone to extract flavonoids, one of the most important functional groups regarding antioxidant strength. However, according to the study's results, it is most likely that other substances/functional groups exist with the same or even higher potential in terms of antioxidant power.

It is also possible to see that these lesser-known plants yielded better inhibition values than several plants that are already used as sources of antioxidant compounds, some present in this study, such as, oregano, basil, rosemary, chamomile, sage, thyme and others (Škrovánková et al.,

2012). Previous studies stated that the plants with the highest antioxidant performance are from the plant's families, *Rosaceae, Empetraceae, Ericaceae, Asteraceae, Lamiaceae, Ginkgoaceae* and many others (Halvorsen et al., 2002), Škrovánková et al., 2012). Some of these families are in the top ten, such as rose hip and wild strawberry (*Rosaceae*); burdock, daisy and milk thristle (*Asteraceae*); heather (*Ericaceae);* white horehound *(Lamiaceae)*. Nevertheless, plants from families less known in this field, as for example sea buckthorn, silver birch or St. John's wort (from the families *Elaeagnaceae, Betulaceae* and *Hypericaceae*, respectively*)* proved in this test to have an even higher potential.

Table 8. DPPH radical inhibition results of the herbs

| No. | Plant Name | Antio. activity E40 /% | Antio. activity E96/% | Antio. activity G75/% |
|---|---|---|---|---|
| 1 | Dried burdock | 65.0 ± 4.0 | 48.3 ± 3.1 | 68.0 ± 1.9 |
| 2 | Couch grass | 63.5 ± 2.6 | 24.3 ± 2.4 | 65.8 ± 2.2 |
| 3 | Silver birch | 75.3 ± 2.0 | 40.7 ± 2.6 | 75.2 ± 1.4 |
| 4 | Sea-buckthorn | 84.0 ± 2.3 | 54.9 ± 3.6 | 72.0 ± 1.8 |
| 5 | Rose hip | 83.7 ± 2.2 | 30.4 ± 3.4 | 81.8 ± 1.3 |
| 6 | Red clover | 66.1 ± 2.7 | 46.7 ± 3.0 | 68.9 ± 2.1 |
| 7 | Common mallow | 68.2 ± 2.7 | 26.7 ± 3.1 | 66.7 ± 2.4 |
| 8 | Elderberry | 67.8 ± 2.4 | 55.8 ± 2.6 | 64.3 ± 2.2 |
| 9 | Galega/ Goat's-rue | 26.3 ± 5.5 | 29.6 ± 2.5 | 23.7 ± 5.8 |
| 10 | European Goldenrod | 41.9 ± 8.2 | 46.3 ± 1.7 | 35.0 ± 3.8 |
| 11 | Hawthorn | 36.2 ± 4.9 | 41.9 ± 2.0 | 43.5 ± 4.3 |
| 12 | Yarrow | 51.2 ± 3.6 | 39.4 ± 1.9 | 52.2 ± 7.8 |
| 13 | Wild Strawberry | 52.3 ± 3.6 | 58.6 ± 1.3 | 61.0 ± 2.7 |
| 14 | Heather | 56.1 ± 4.2 | 59.9 ± 1.2 | 54.3 ± 8.5 |
| 15 | Dandelion | 36.0 ± 6.4 | 30.4 ± 2.9 | 45.1 ± 5.1 |
| 16 | Common Soapwort | 46.3 ± 4.5 | 11.9 ± 3.6 | 47.4 ± 3.1 |
| 17 | Common hop | 77.2 ± 1.7 | 79.6 ± 1.6 | 71.7 ± 2.1 |
| 18 | Garlic | 42.3 ± 5.8 | 11.7 ± 3.0 | 41.9 ± 3.5 |
| 19 | Small-leaved lime | 68.2 ± 1.8 | 56.9 ± 3.4 | 65.9 ± 1.4 |
| 20 | Yellow sweet clover | 66.2 ± 4.7 | 34.3 ± 5.8 | 58.1 ± 1.4 |
| 21 | Wormwood | 66.9 ± 1.7 | 44.1 ± 4.7 | 59.9 ± 1.5 |
| 22 | St John's-wort | 76.1 ± 2.0 | 52.2 ± 4.1 | 74.4 ± 1.2 |
| 23 | Dandelion | 64.1 ± 2.1 | 48.2 ± 4.2 | 55.4 ± 2.0 |
| 24 | Motherwort | 62.4 ± 2.2 | 44.5 ± 5.0 | 54.3 ± 2.3 |
| 25 | Common comfrey | 43.8 ± 4.1 | 51.8 ± 4.4 | 48.9 ± 1.7 |
| 26 | Daisy | 71.9 ± 1.9 | 48.6 ± 4.5 | 70.3 ± 1.8 |

*At green, are the top 5 herbal extracts and at yellow there are the next 5 herbal extracts at top 10 in terms of inhibition of DPPH radical.

Table 8. DPPH radical inhibition results of the herbs (continuation)

| No. | Plant Name | Antio. activity E40/%* | Antio. activity E96/%* | Antio. activity G75/%* |
|---|---|---|---|---|
| 27 | Lingonberry | 35.1 ± 4.8 | 25.4 ± 6.5 | 24.7 ± 1.9 |
| 28 | Urtica | 10.6 ± 7.2 | 11.7 ± 8.2 | 0.0 ± 4.7 |
| 29 | Hemp nettle | 21.1 ± 5.1 | 3.2 ± 8.5 | 7.3 ± 3.2 |
| 30 | Basil | 56.0 ± 2.6 | 25.5 ± 5.9 | 39.4 ± 2.3 |
| 31 | Ramson, Wild garlic | 44.5 ± 4.1 | 7.0 ± 9.4 | 29.4 ± 3.0 |
| 32 | Veronica | 40.8 ± 3.1 | 13.5 ± 6.8 | 21.4 ± 2.0 |
| 33 | Ground-ivy | 45.9 ± 3.1 | 20.5 ± 6.4 | 33.1 ± 2.6 |
| 34 | Common Agrimony | 59.0 ± 4.6 | 33.5 ± 5.3 | 45.2 ± 2.8 |
| 35 | Valerian | 68.4 ± 1.8 | 36.5 ± 5.4 | 62.6 ± 1.1 |
| 36 | White horehound | 65.3 ± 3.5 | 50.3 ± 3.2 | 59.1 ± 3.0 |
| 37 | Dyer's greenweed | 67.2 ± 3.8 | 41.5 ± 3.7 | 63.3 ± 1.5 |
| 38 | Liquorice | 42.2 ± 5.9 | 41.5 ± 3.9 | 37.2 ± 3.7 |
| 39 | Horsetail | 64.4 ± 4.2 | 48.3 ± 3.4 | 57.3 ± 2.3 |
| 40 | Buckwheat | 66.8 ± 3.7 | 48.2 ± 3.3 | 61.3 ± 3.1 |
| 41 | Calendula | 65.1 ± 3.9 | 27.7 ± 5.4 | 62.1 ± 2.2 |
| 42 | Sage | 43.2 ± 6.1 | 32.5 ± 4.2 | 50.3 ± 2.7 |
| 43 | Breckland thyme | 59.8 ± 4.4 | 44.8 ± 4.4 | 49.3 ± 2.4 |
| 44 | Oregano | 41.3 ± 6.4 | 10.6 ± 6.3 | 28.9 ± 2.8 |
| 45 | Willow | 59.4 ± 4.8 | 35.2 ± 4.7 | 37.9 ± 2.5 |
| 46 | Rosemary | 40.4 ± 6.6 | 17.2 ± 6.3 | 53.8 ± 2.1 |
| 47 | Chamomile | 57.0 ± 5.0 | 16.0 ± 5.6 | 59.1 ± 2.5 |
| 48 | Elder flower | 50.6 ± 6.8 | 0.0 ± 6.8 | 51.9 ± 3.6 |
| 49 | Wall germander | 48.5 ± 5.5 | 12.4 ± 6.1 | 53.1 ± 1.9 |
| 50 | White Mustard | 61.6 ± 4.9 | 12.5 ± 5.8 | 52.2 ± 5.2 |
| 51 | Common chicory | 51.2 ± 5.1 | 15.0 ± 5.6 | 49.2 ± 3.5 |
| 52 | Milk thistle | 65.9 ± 3.7 | 48.4 ± 3.7 | 59.7 ± 2.7 |

*At green, are the top 5 herbal extracts and at yellow are the next 5 herbal extracts at top 10 in terms of inhibition of DPPH radical.

## 6.4. Flavonoids' Quantification test

For the quantification of flavonoids, it was also used a chromatographic method, aluminium chloride ($AlCl_3$) colorimetric assay, with rutin as flavonoids' equivalent (Appendix B). To this end, a calibration curve of rutin solutions (0-5 g/L) was created. The results of the flavonoids' quantification can be seen in Table 9 for each solvent's extraction method (the results can be seen in graphs in Appendix D).

The tables present in green the top five herbs and at yellow the next five herbs of the top ten in terms of flavonoids content, for each solvent. The best results, in general, were also obtained in the 40 % EtOH and 75 % glycerol extracts (6000-17000 mg/L), whereby in some herbs the best was the alcoholic extracts and in another cases the glycerol extracts. In terms of flavonoids' content, there are some herbs worth mentioning such as, wild strawberry, lingonberry, common agrimony, liquorice, willow, rosemary, and wall germander as exceptional sources of flavonoids.

Analysing the Table 9 in correlation with the data of the Table 8 it was possible to correlate three different scenarios of interpretation.

In the first scenario, there is no relation between the quantity of flavonoids and the percentage of antioxidant potential, or if it exists it has little impact on the overall activity of the extracts. To demonstrate this line of thought we have the example of the European goldenrod, where the best result of antioxidant inhibition does not correspond to the highest content of flavonoids, in the same conditions of extraction (corresponding solvent). Besides the European goldenrod, there are other plants in this study in the same situation such as couch grass, sea-buckthorn, galega, hawthorn, wild strawberry, heather, dandelion (No. 23), common comfrey, lingonberry, urtica, hempnettle, ramson, liquorice, horsetail, white mustard and milk thistle.

In the second scenario, it is clearly noticeable a relation between the quantity of flavonoids and the percentage of antioxidant inhibition. For the clarification of this scenario, we have the example of dried burdock, where

the highest percentage of DPPH inhibition is aligned with the highest quantity of flavonoids, and normally, with the decrease of the quantity of flavonoids there is a decrease in the inhibition percentage (more clear comparing 40 % EtOH and 75 % glycerol extracts). Naturally, taking into account the sample size, there are other herbs in the same situation, such as rose hip, red clover, common soapwort, yellow sweet clover, wormwood, motherwort and rosemary. However, it is almost certain that there are other compounds in the solvent matrix also with antioxidant potential, nevertheless in this scenario the major impact of the antioxidant potential is directed to the quantity of flavonoids.

In the third scenario, it is possible to make a relation between the quantity of flavonoids and the percentage of antioxidant inhibition, but not as clear as the second scenario. In this scenario, it is noticeable that, as the amount of flavonoids increases, there is an increase of the inhibition percentage. However, that increase is not as proportional as it was in the previous scenario. In this instance, it is possible the involvement of other substances with the same or even higher relevance, in terms of antioxidant potential. In order to demonstrate this situation, we have the example of elderberry, where the 96 % EtOH extracts (less prone to extract flavonoids) have half of the concentration of the flavonoids of 40 % EtOH extracts with just a decrease of 10 % of antioxidant inhibition. Other than elderberry, there are other herbs in the same position, such as silver birch, common mallow, yarrow, dandelion (No.15), common hop, garlic, small-leaved lime, S. John's wort, daisy, basil, veronica, ground-ivy, common agrimony, valerian, white horehound, Dyer's greenweed, buckwheat, calendula, sage, breckland thyme, oregano, willow, chamomile, elderflower, wall germander and chicory.

Aside from these scenarios, it is important to note that, in a generalised way, alcohol (EtOH) reduces the levels of action of antioxidant agents, as stated by Wu and Cederbaum (2009). Therefore, it is normal to have a reduction of the antioxidant potential, as is the case of white mustard, where the quantity of flavonoids is quite similar in the three

extraction methods, but the 96 % EtOH extracts have a decrease of sensitively 40-50 % of DPPH inhibition. Clearly, this phenomenon is occurring with all the herbs, each one with more or less interference, depending on the sensitivity of the substances that have the biological potential.

Even though some herbs are a great source of flavonoids, but even without any relevance in terms of antioxidant potential, they can be applied in other markets. In this selection are some herbs that had been reported to have a great potential for the cosmetic market, with antiaging properties (Sousa, 2022). This is the case for wild strawberry, lingonberry, common agrimony, liquorice, breckland thyme and willow (Sousa, 2022).

Table 9. Results of flavonoids' quantification test for each solvent's extraction

| No. | Plant Name | Flavonoids /(mg/L) E40 * | Flavonoids /(mg/L) E96* | Flavonoids /(mg/L) G75* |
|---|---|---|---|---|
| 1 | Dried burdock | 1230 ± 89 | 239 ± 93 | 1579 ± 42 |
| 2 | Couch grass | 462 ± 91 | 244 ± 93 | 307 ± 43 |
| 3 | Silver birch | 4531 ± 106 | 2511 ± 90 | 3568 ± 46 |
| 4 | Sea-buckthorn | 1673 ± 89 | 1198 ± 89 | 234 ± 44 |
| 5 | Rose hip | 3462 ± 96 | 987 ± 89 | 3310 ± 45 |
| 6 | Red clover | 2636 ± 91 | 256 ± 92 | 3518 ± 46 |
| 7 | Common mallow | 1520 ± 89 | 711 ± 90 | 1756 ± 42 |
| 8 | Elderberry | 3465 ± 96 | 1648 ± 89 | 3176 ± 44 |
| 9 | Galega/ Goat's-rue | 1535 ± 89 | 927 ± 90 | 1437 ± 42 |
| 10 | European Goldenrod | 4932 ± 111 | 1461 ± 89 | 3871 ± 47 |
| 11 | Hawthorn | 7235 ± 90 | 2355 ± 90 | 6703 ± 42 |
| 12 | Yarrow | 4760 ± 109 | 1187 ± 89 | 3556 ± 46 |
| 13 | Wild Strawberry | 10092 ± 89 | 3683 ± 98 | 10683 ± 42 |
| 14 | Heather | 7327 ± 90 | 3090 ± 93 | 3631 ± 46 |
| 15 | Dandelion | 358 ± 92 | 399 ± 92 | 442 ± 43 |
| 16 | Common Soapwort | 806 ± 90 | 353 ± 92 | 849 ± 42 |
| 17 | Common hop | 2827 ± 92 | 2378 ± 90 | 2969 ± 44 |
| 18 | Garlic | 198 ± 93 | 36 ± 94 | 120 ± 44 |
| 19 | Small-leaved lime | 2828 ± 92 | 1211 ± 89 | 3812 ± 47 |
| 20 | Yellow sweet clover | 2172 ± 89 | 937 ± 90 | 1733 ± 42 |
| 21 | Wormwood | 1852 ± 89 | 884 ± 90 | 1484 ± 42 |
| 22 | St John's-wort | 4627 ± 107 | 1573 ± 89 | 4059 ± 48 |
| 23 | Dandelion | 1973 ± 89 | 786 ± 90 | 2726 ± 43 |
| 24 | Motherwort | 1331 ± 89 | 567 ± 91 | 1152 ± 42 |
| 25 | Common comfrey | 1863 ± 89 | 193 ± 93 | 2978 ± 44 |
| 26 | Daisy | 4025 ± 101 | 1177 ± 89 | 2979 ± 44 |

* At green, are the top 5 herbal extracts and at yellow are the next 5 herbal extracts at top 10 in terms in terms of flavonoids content

Table 9. Results of flavonoids' quantification test for each solvent's extraction (continuation)

| No. | Plant Name | Flavonoids /(mg/L) E40 * | Flavonoids /(mg/L) E96* | Flavonoids /(mg/L) G75* |
|---|---|---|---|---|
| 27 | Lingonberry | 16944 ± 89 | 7344 ± 98 | 15188 ± 42 |
| 28 | Urtica | 574 ± 91 | 657 ± 91 | 921 ± 42 |
| 29 | Hempnettle | 2821 ± 92 | 1184 ± 89 | 2727 ± 43 |
| 30 | Basil | 6210 ± 90 | 1451 ± 89 | 6009 ± 43 |
| 31 | Ramson, Wild garlic | 1462 ± 89 | 1646 ± 89 | 1445 ± 42 |
| 32 | Veronica | 4468 ± 105 | 1484 ± 89 | 4150 ± 48 |
| 33 | Ground-ivy | 2500 ± 90 | 999 ± 89 | 2378 ± 42 |
| 34 | Common Agrimony | 8585 ± 89 | 2980 ± 93 | 7788 ± 42 |
| 35 | Valerian | 931 ± 90 | 687 ± 90 | 1147 ± 42 |
| 36 | White horehound | 2496 ± 90 | 669 ± 90 | 1835 ± 42 |
| 37 | Dyer's greenweed | 4756 ± 109 | 2757 ± 92 | 2506 ± 43 |
| 38 | Liquorice | 9273 ± 89 | 4272 ± 103 | 5576 ± 43 |
| 39 | Horsetail | 584 ± 91 | 851 ± 90 | 786 ± 42 |
| 40 | Buckwheat | 1909 ± 89 | 1142 ± 89 | 1404 ± 42 |
| 41 | Calendula | 3085 ± 93 | 1175 ± 89 | 2405 ± 42 |
| 42 | Sage | 6129 ± 90 | 2878 ± 92 | 6313 ± 42 |
| 43 | Breckland thyme | 7685 ± 89 | 2287 ± 90 | 6275 ± 42 |
| 44 | Oregano | 6514 ± 90 | 1181 ± 89 | 6329 ± 42 |
| 45 | Willow | 13003 ± 89 | 4123 ± 102 | 2689 ± 43 |
| 46 | Rosemary | 7701 ± 89 | 2342 ± 90 | 13675 ± 42 |
| 47 | Chamomile | 3286 ± 95 | 969 ± 89 | 2004 ± 42 |
| 48 | Elder flower | 6226 ± 90 | 1394 ± 89 | 4287 ± 49 |
| 49 | Wall germander | 12949 ± 89 | 4335 ± 104 | 8969 ± 42 |
| 50 | White Mustard | 908 ± 90 | 728 ± 90 | 812 ± 42 |
| 51 | Common chicory | 562 ± 91 | 385 ± 92 | 589 ± 43 |
| 52 | Milk thistle | 2310 ± 90 | 2864 ± 92 | 635 ± 43 |

* At green, are the top 5 herbal extracts and at yellow are the next 5 herbal extracts at top 10 in terms in terms of flavonoids content

## 6.5. Vitamin C equivalents

Beyond the presentation study of the herbal extract's functionality, they can also be presented in a more commercial form, in order to exhibit their potential compared to those products that are already in the market. In order to do that, the extracts were converted in vitamin C equivalents (Appendix A), as can be seen in Table 10, for each solvent.

From a first glance, it is possible to see that in general the best results are found in the E40 and G75 extracts, since the best results of antioxidant potential are as well from these extraction options. The most interesting herbs continue to be silver birch, sea-buckthorn, rose hip, common hop and St. John's wort for the E40 and G75 extracts, and sea-buckthorn, elderberry, wild strawberry, heather and common hop for the E96 extracts. Nevertheless, there are more options to consider in the top ten (or more).

Nearly all the best results of extractions present concentrations above 400 mg/L of vitamin C equivalents, however these extracts can still be concentrated so they can be presented in a more competitive form on the market (most products currently on the market have a concentration of 1000 mg of vitamin C).

Besides the supplementation applications, these extracts have also the possibility of being applied in other markets, as for example the smart packaging market. The antioxidant functionality can prevent oxidation reactions with the application of these extracts in the packaging films, thus improving the stability of food products and extending their shelf's life (Biji et al., 2015). Additionally, there is also the possibility of acting as flavour or odour absorbers and releasers in the control of amines' oxidation reactions, responsible for unpleasant flavours and odours (Biji et al., 2015).

Table 10. Results of Vitamin C equivalents test for each solvent's extraction

| No. | Plant Name | Vit. C eq. E40 /mg/L* | Vit. C eq. E96 /mg/L* | Vit. C eq. G75 /mg/L* |
|---|---|---|---|---|
| 1 | Dried burdock | 388 ± 16 | 316 ± 10 | 399 ± 7 |
| 2 | Couch grass | 382 ±10 | 218 ± 5 | 390 ± 9 |
| 3 | Silver birch | 429 ± 9 | 285 ± 7 | 428 ± 6 |
| 4 | Sea-buckthorn | 464 ± 11 | 343 ± 12 | 415 ± 7 |
| 5 | Rose hip | 462 ± 10 | 243 ± 8 | 454 ± 6 |
| 6 | Red clover | 393 ± 10 | 309 ± 9 | 403 ± 8 |
| 7 | Common mallow | 401 ± 11 | 228 ± 7 | 394 ± 9 |
| 8 | Elderberry | 399 ± 10 | 347 ± 9 | 384 ± 9 |
| 9 | Galega/ Goat's-rue | 235 ± 13 | 239 ± 6 | 222 ± 13 |
| 10 | European Goldenrod | 297 ± 24 | 308 ± 5 | 267 ± 10 |
| 11 | Hawthorn | 274 ± 13 | 290 ± 6 | 301 ± 13 |
| 12 | Yarrow | 334 ± 12 | 279 ± 5 | 336 ± 26 |
| 13 | Wild Strawberry | 338 ± 12 | 358 ± 5 | 371 ± 10 |
| 14 | Heather | 353 ± 15 | 363 ± 4 | 344 ± 29 |
| 15 | Dandelion | 274 ± 17 | 243 ± 7 | 308 ± 16 |
| 16 | Common Soapwort | 314 ± 14 | 167 ± 6 | 317 ± 10 |
| 17 | Common hop | 436 ± 8 | 444 ± 7 | 414 ± 9 |
| 18 | Garlic | 298 ± 17 | 166 ± 5 | 295 ± 10 |
| 19 | Small-leaved lime | 402 ± 7 | 342 ± 12 | 395 ± 5 |
| 20 | Yellow sweet clover | 395 ± 18 | 244 ± 14 | 364 ± 5 |
| 21 | Wormwood | 397 ± 7 | 287 ± 13 | 371 ± 6 |
| 22 | St John's-wort | 433 ± 9 | 321 ± 13 | 427 ± 5 |
| 23 | Dandelion | 386 ± 8 | 304 ± 13 | 353 ± 7 |
| 24 | Motherwort | 380 ± 9 | 288 ± 14 | 349 ± 8 |
| 25 | Common comfrey | 307 ± 13 | 320 ± 14 | 328 ± 6 |
| 26 | Daisy | 417 ± 8 | 306 ± 14 | 412 ± 8 |

*At green, are the top 5 herbal extracts and at yellow are the next 5 herbal extracts at top 10 in terms in terms of vitamin C' equivalents

Table 10. Results of Vitamin C equivalents test for each solvent's extraction (continuation)

| No. | Plant Name | Vit. C eq. E40 /mg/L* | Vit. C eq. E96 /mg/L* | Vit. C eq. G75 /mg/L* |
|---|---|---|---|---|
| 27 | Lingonberry | 273 ± 13 | 206 ± 13 | 234 ± 4 |
| 28 | Urtica | 88 ± 6 | 147 ± 12 | 0 ± 1 |
| 29 | Hemp nettle | 219 ± 11 | 71 ± 6 | 167 ± 5 |
| 30 | Basil | 355 ± 9 | 207 ± 12 | 291 ± 7 |
| 31 | Ramson, Wild garlic | 310 ± 13 | 7 ± 1 | 253 ± 7 |
| 32 | Veronica | 295 ± 9 | 155 ± 11 | 222 ± 4 |
| 33 | Ground-ivy | 315 ± 10 | 185 ± 12 | 267 ± 7 |
| 34 | Common Agrimony | 366 ± 17 | 241 ± 13 | 314 ± 9 |
| 35 | Valerian | 403 ± 7 | 254 ± 14 | 382 ± 4 |
| 36 | White horehound | 403 ± 14 | 328 ± 11 | 386 ± 12 |
| 37 | Dyer's greenweed | 410 ± 16 | 293 ± 11 | 401 ± 6 |
| 38 | Liquorice | 321 ± 19 | 293 ± 11 | 311 ± 12 |
| 39 | Horsetail | 400 ± 17 | 321 ± 11 | 380 ± 9 |
| 40 | Buckwheat | 409 ± 15 | 320 ± 10 | 394 ± 12 |
| 41 | Calendula | 403 ± 16 | 238 ± 13 | 397 ± 9 |
| 42 | Sage | 325 ± 20 | 257 ± 11 | 356 ± 10 |
| 43 | Breckland thyme | 384 ± 17 | 307 ± 14 | 353 ± 8 |
| 44 | Oregano | 318 ± 20 | 170 ± 11 | 283 ± 8 |
| 45 | Willow | 383 ± 19 | 268 ± 13 | 313 ± 8 |
| 46 | Rosemary | 315 ± 21 | 196 ± 12 | 368 ± 8 |
| 47 | Chamomile | 374 ± 19 | 192 ± 11 | 387 ± 10 |
| 48 | Elder flower | 351 ± 24 | 120 ± 8 | 362 ± 13 |
| 49 | Wall germander | 344 ± 19 | 177 ± 11 | 366 ± 7 |
| 50 | White Mustard | 390 ± 19 | 178 ± 10 | 363 ± 19 |
| 51 | Common chicory | 353 ± 18 | 188 ± 11 | 352 ± 12 |
| 52 | Milk thistle | 406 ± 15 | 321 ± 12 | 388 ± 11 |

*At green, are the top 5 herbal extracts and at yellow are the next 5 herbal extracts at top 10 in terms in terms of vitamin C' equivalents

## 6.6. Dry Weight Measurement

For the determination of dry weight of the alcoholic extracts, a specific quantity of extract was evaporated in order to determine the concentration (in g/L) of the total bioactive compounds (Appendix F). Correlating these values with the data of flavonoids' equivalents, as mentioned earlier in the methodology, it is possible to obtain the percentage of flavonoids in the herbal extracts, for each plant. Figures 4 and 5 present these percentages for each solvent.

Firstly, it is possible to notice more clearly the composition of the extracts, where in this case, 8 herbs of E40 extracts and 7 herbs of E96 extracts have more than 40 % of the total bioactive substances, in flavonoids. However, most of the plants have between 10-40 % of flavonoids in its composition.

These data will also help to explain and determine the possibility of other functional groups interfering on the antioxidant activity, as discussed earlier. As an exemplification, sea-buckthorn only have 5.9 % of flavonoids on the E40 extracts, which implies that it is very likely that other functional groups be responsible for the antioxidant activity, for example the polyphenols or even others. On the other hand, with this information, it is also feasible to check its non-relatedness, as for the example of lingonberry that has a relatively small percentage of antioxidant inhibition in contradiction to the more than 60 % of flavonoids in E40 extracts, reinforcing its non-relationship.

Finally, as can be seen in Appendix F, the concentration of bioactive compounds is in general higher in the E40 extracts than the E96 extracts, however, it is important to state that this does happen not for every type of substance, since every functional group has a different affinity to the polarity of the solvents which extract them. For example, in this particular study, it was possible to notice that flavonoids have a greater affinity to the 40 % EtOH solvent.

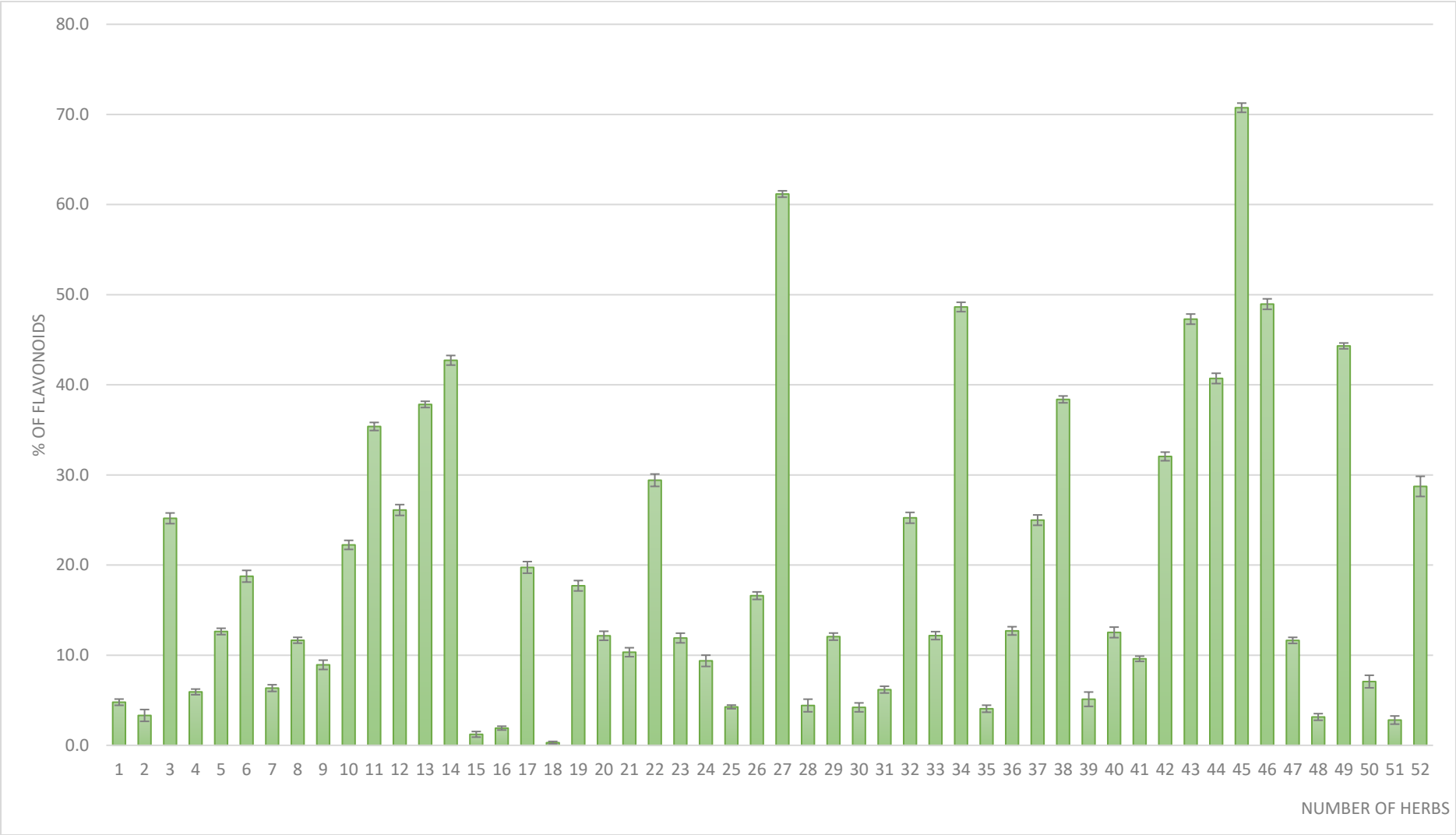


Figure 4. Percentage of flavonoids in the E40 extracts.

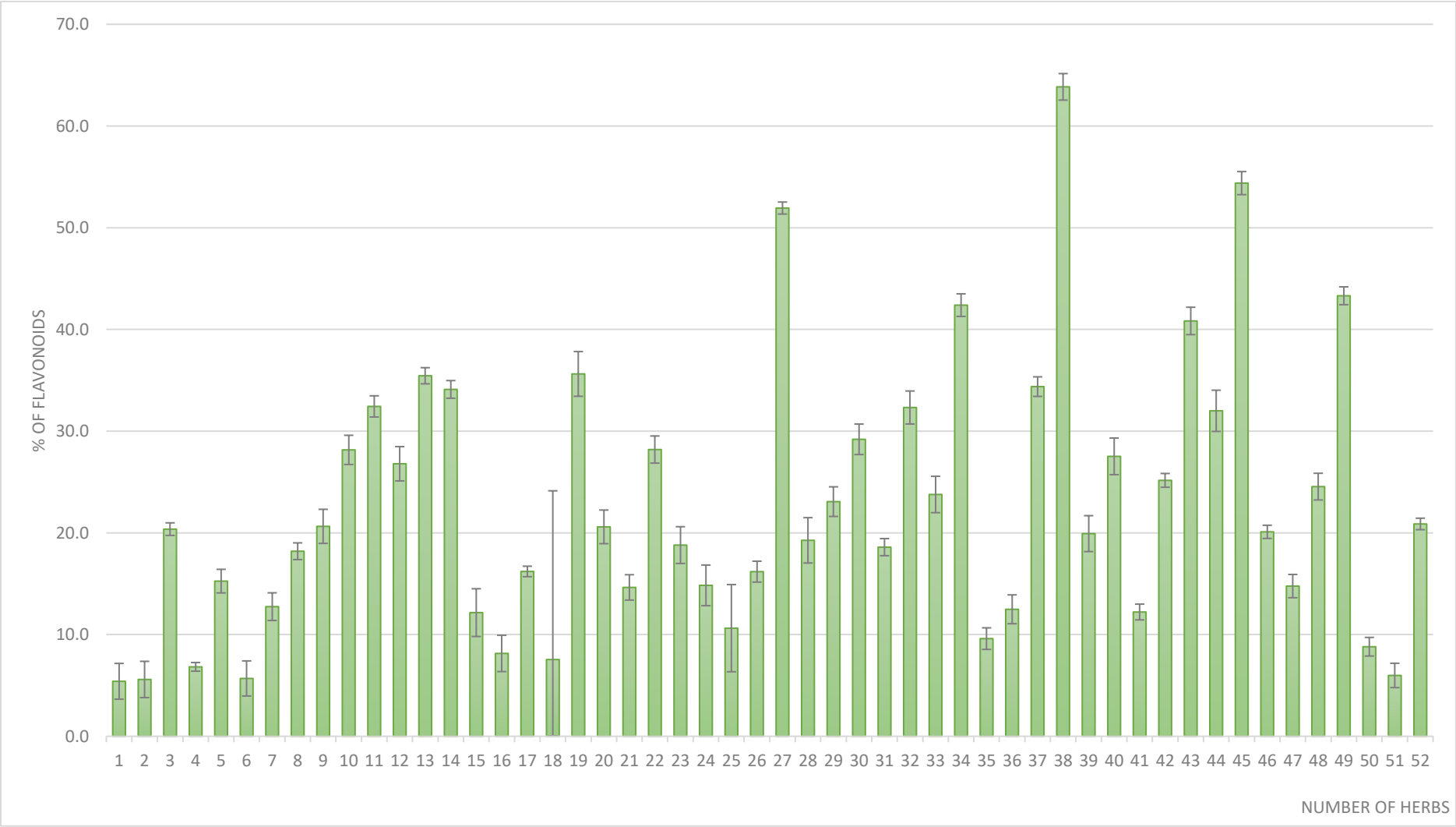


Figure 5. Percentage of flavonoids in the E96 extracts.

## 6.7. Optimization process

### 6.7.1. Common Hop

After data collection (Appendix E), the software Design Expert (SDE) analysed the information and presented the mathematical models that best fit the existing data. Figure 6, shows the Fit Summary as calculated by the program.

**Fit Summary**

**Response 1: Flavonoids**

| | Source | Sequential p-value | Lack of Fit p-value | Adjusted $R^2$ | Predicted $R^2$ | |
|---|---|---|---|---|---|---|
| | **Linear** | **0.0010** | **0.0031** | **0.6056** | **0.3911** | **Suggested** |
| | 2FI | 0.6866 | 0.0020 | 0.5427 | -6.2789 | |
| | **Quadratic** | **0.0047** | **0.0238** | **0.9405** | **-1.8900** | **Suggested** |
| | Cubic | 0.0238 | | 0.9918 | | Aliased |

Figure 6. Fit Summary of common hop experiment.

This information clearly conveys that only the linear and quadratic models are relatively reliable. For the 2FI model, it has a significantly high sequential *p*-value and for a, model to be accepted it must have, in the worst scenario, a value equal to 0.05, at most. The cubic model in this case is aliased, then it cannot accurately fit the design and should not be considered for analysis. Nevertheless, this statistical study is not sufficient to validate the choice of a model to represent the data, thus an ANOVA test was conducted for each of the two suggested models. The results for each model can be seen in Figures 7 and 8.

From the ANOVA test results of the linear and quadratic models, it is possible to state that none of the models are accurate mathematical

representations of the data. This position is declared by the value of the Lack of Fit F-value for the residuals of 59.77 and 16.61 respectively, which is in both cases significant. In other words, there is only 3.1 % or 2.8 % chance that a Lack of Fit F-value this large could occur due to noise, respectively. These values state that the presented data are not well represented by either the linear or the quadratic model. For the model to be an actual representation of the information, the lack of fit value must be not significant, which means that the *p*-value must be higher than 0.10.

Beyond that, it is also possible to see from this analysis that there are some model terms that have non-significant *p*-values (*p*-value greater than 0.10). If there are many non-significant model terms, a model reduction could improve the model itself. For example, in the quadratic model there are five non-significant model terms (A, AC, BC, CD and $A^2$). The model term of temperature has by itself a high *p*-value, but nevertheless, it is an important parameter to study. From other perspective, it is possible to notice that the other model terms are all interactions with the model term C, time. Being the extraction process proceeded by maceration, it is quite obvious that, the longer the extraction time, the greater the quantity of flavonoids extracted. Therefore, the removal of the model term, time, could improve the accuracy of the mathematical model, enabling the possibility for an actual mathematical representation of the data.

**ANOVA for Linear model**

**Response 1: Flavonoids**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 3.077E+06 | 4 | 7.693E+05 | 8.29 | 0.0010 | significant |
| A-Temperature | 2.156E+05 | 1 | 2.156E+05 | 2.32 | 0.1482 | |
| B-Ratio | 1.474E+06 | 1 | 1.474E+06 | 15.89 | 0.0012 | |
| C-Time | 3.110E+05 | 1 | 3.110E+05 | 3.35 | 0.0870 | |
| D-Solvent concentration | 1.080E+06 | 1 | 1.080E+06 | 11.64 | 0.0039 | |
| **Residual** | 1.392E+06 | 15 | 92778.12 | | | |
| Lack of Fit | 1.386E+06 | 12 | 1.155E+05 | 59.77 | 0.0031 | significant |
| Pure Error | 5796.75 | 3 | 1932.25 | | | |
| **Cor Total** | 4.469E+06 | 19 | | | | |

Figure 7. ANOVA test for the linear model of common hop experiment.

**ANOVA for Quadratic model**

**Response 1: Flavonoids**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 4.399E+06 | 14 | 3.142E+05 | 22.45 | 0.0014 | significant |
| A-Temperature | 161.18 | 1 | 161.18 | 0.0115 | 0.9187 | |
| B-Ratio | 8.661E+05 | 1 | 8.661E+05 | 61.87 | 0.0005 | |
| C-Time | 2.393E+05 | 1 | 2.393E+05 | 17.10 | 0.0090 | |
| D-Solvent concentration | 6.526E+05 | 1 | 6.526E+05 | 46.62 | 0.0010 | |
| AB | 1.316E+05 | 1 | 1.316E+05 | 9.40 | 0.0279 | |
| AC | 3099.15 | 1 | 3099.15 | 0.2214 | 0.6578 | |
| AD | 1.960E+05 | 1 | 1.960E+05 | 14.00 | 0.0134 | |
| BC | 0.1297 | 1 | 0.1297 | 9.268E-06 | 0.9977 | |
| BD | 66338.88 | 1 | 66338.88 | 4.74 | 0.0814 | |
| CD | 44315.75 | 1 | 44315.75 | 3.17 | 0.1353 | |
| $A^2$ | 946.50 | 1 | 946.50 | 0.0676 | 0.8052 | |
| $B^2$ | 1.297E+05 | 1 | 1.297E+05 | 9.27 | 0.0286 | |
| $C^2$ | 3.333E+05 | 1 | 3.333E+05 | 23.81 | 0.0046 | |
| $D^2$ | 1.636E+05 | 1 | 1.636E+05 | 11.69 | 0.0189 | |
| **Residual** | 69996.03 | 5 | 13999.21 | | | |
| Lack of Fit | 64199.28 | 2 | 32099.64 | 16.61 | 0.0238 | significant |
| Pure Error | 5796.75 | 3 | 1932.25 | | | |
| **Cor Total** | 4.469E+06 | 19 | | | | |

Figure 8. ANOVA test for the quadratic model of common hop experiment.

### 6.7.2. Sea-Buckthorn

Figure 9, shows the Fit Summary that the software Design Expert has calculated with the experimental data of sea-buckthorn (Appendix E).

**Fit Summary**

**Response 1: Flavonoids**

| Source | Sequential p-value | Lack of Fit p-value | Adjusted $R^2$ | Predicted $R^2$ | |
|---|---|---|---|---|---|
| **Linear** | **< 0.0001** | **0.1774** | **0.8696** | **0.7759** | **Suggested** |
| **2FI** | **0.0179** | **0.4827** | **0.9485** | **0.7647** | **Suggested** |
| Quadratic | 0.6615 | 0.2991 | 0.9384 | -1.0766 | |
| Cubic | 0.2991 | | 0.9541 | | Aliased |

Figure 9. Fit Summary of sea-buckthorn experiment.

In the sea-buckthorn case, only the linear and 2FI models are suggested as possible models to represent the data. Even though the $R^2$ of the quadratic model is quite good, it is important to notice that the model does not have a significant sequential *p*-value, whereby, the model cannot be accepted. For the cubic model, as mentioned earlier, the model is aliased, therefore it cannot be an option of representation. Nevertheless, as stated before, an ANOVA test for both models is still required. The results for each model can be seen in Figures 10 and 11.

The results of the ANOVA tests show that the linear and the 2FI models have the premises to be mathematical representations of the collected data. This conclusion is supported by the fact that both models have a non-significant Lack of Fit *p*-value, in other words, there is only 17.74 % or 48.27 % chance that a Lack of Fit F-value this large could occur due to noise, respectively. With this statistical information, it is possible to state that both models can be mathematical representations of the experimental data.

Despite the fact that both models have the statistical requirements to represent the experimental data, the 2FI model had the best results in

mathematical terms. This means that the 2FI model presents a more accurate model, with a higher $R^2$ and a Lack of Fit *p*-value bigger, representing more correctly the technical information. Beyond that, only the 2FI and quadratic model have the possibility to present the interactions of the input variables providing more data to the optimization process deliberation.

**ANOVA for 2FI model**

**Response 1: Flavonoids**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 1.241E+07 | 10 | 1.241E+06 | 35.96 | < 0.0001 | significant |
| A-Temperature | 86418.39 | 1 | 86418.39 | 2.51 | 0.1479 | |
| B-Ratio | 2.027E+06 | 1 | 2.027E+06 | 58.76 | < 0.0001 | |
| C-Time | 1.924E+06 | 1 | 1.924E+06 | 55.78 | < 0.0001 | |
| D-Solvent concentratiom | 2.264E+05 | 1 | 2.264E+05 | 6.56 | 0.0306 | |
| AB | 1.777E+05 | 1 | 1.777E+05 | 5.15 | 0.0494 | |
| AC | 1.769E+05 | 1 | 1.769E+05 | 5.13 | 0.0498 | |
| AD | 2.941E+05 | 1 | 2.941E+05 | 8.53 | 0.0170 | |
| BC | 64253.12 | 1 | 64253.12 | 1.86 | 0.2055 | |
| BD | 62766.53 | 1 | 62766.53 | 1.82 | 0.2103 | |
| CD | 1.909E+05 | 1 | 1.909E+05 | 5.53 | 0.0431 | |
| **Residual** | 3.105E+05 | 9 | 34497.31 | | | |
| Lack of Fit | 2.182E+05 | 6 | 36372.51 | 1.18 | 0.4827 | not significant |
| Pure Error | 92240.75 | 3 | 30746.92 | | | |
| **Cor Total** | 1.272E+07 | 19 | | | | |

Figure 10. ANOVA test for the 2FI model of sea-buckthorn experiment.

**ANOVA for Linear model**

**Response 1: Flavonoids**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 1.141E+07 | 4 | 2.852E+06 | 32.66 | < 0.0001 | significant |
| A-Temperature | 1.343E+06 | 1 | 1.343E+06 | 15.38 | 0.0014 | |
| B-Ratio | 4.547E+06 | 1 | 4.547E+06 | 52.08 | < 0.0001 | |
| C-Time | 1.905E+06 | 1 | 1.905E+06 | 21.82 | 0.0003 | |
| D-Solvent concentratiom | 3.625E+06 | 1 | 3.625E+06 | 41.53 | < 0.0001 | |
| **Residual** | 1.310E+06 | 15 | 87303.97 | | | |
| Lack of Fit | 1.217E+06 | 12 | 1.014E+05 | 3.30 | 0.1774 | not significant |
| Pure Error | 92240.75 | 3 | 30746.92 | | | |
| **Cor Total** | 1.272E+07 | 19 | | | | |

Figure 11. ANOVA test for the linear model of sea-buckthorn experiment.

In Figures 12-17 it is possible to see all the model terms' interactions that the software created. These model graphs provide very important information, regarding which variables are more important for the maximization of flavonoids' extraction.

Figure 12 portrays the interaction of solvent ratio and temperature. Through its analysis it was possible to see that, flavonoids concentrate more in lower ratios, in this particular case really near the optimal minimal ratio, already mentioned in Table 6. Besides that, it is also possible to note that flavonoids concentrations are slightly higher at lower temperatures, even after the fact that the peak of flavonoids quantity in Figure 13 is at higher temperatures. This situation possibly means that, concerning flavonoids extraction, the solvent ratio plays a more significant factor than temperature.

In Figure 13, it is possible to see the interaction of the temperature and time. From its analysis, it is clear that flavonoids' extraction is as greater as longer the time of extraction, and this logic can be applied in all model terms' interactions. From Figure 13, it is also possible to conclude

that flavonoids are better extracted at higher temperatures, 40 ºC, without the interaction of other factors.

In Figure 14, the graph shows the interaction of solvent concentration of EtOH and temperature. From a first impression, it is quite clear that flavonoids' extraction is more prone to occur at lower concentrations of EtOH, specially at percentages of 40-56 %, in this particular case. However, in contrast to Figure 13, the highest quantity is extracted at higher temperatures, whereby the solvent concentration does not behave in competitive way with the variable, temperature.

In Figure 15, the interaction of time and solvent ratio confirms once again that the extraction of flavonoids is significantly higher at lower ratios. However, from this graph that the conclusion is best supported, since it is possible to observe the proportionality of decay through the ratio growth.

Figure 16 presents the relation between solvent ratio and solvent percentage of EtOH, and once more the highest extraction of flavonoids is carried near the minimal optimal ratio zone, even in high percentages of alcohol. Beyond that, as stated in Figure 14, lower percentages of EtOH promote more extraction of flavonoids, in this case between 40-69 %, flavonoids' concentration is higher than 4000 mg/L.

Finally, in Figure 17 the model terms interaction of solvent concentration and time, comes once more proving that the extraction of flavonoids is much more susceptible at lower concentrations of EtOH, where this particular graph shows that the concentration of flavonoids is higher than 3500 mg/L between 40-63 % of alcohol.

Therefore, through the analysis of these six graphs, the most advantageous conditions for flavonoids extraction of the herb sea-buckthorn are a temperature near 40 ºC, a solvent ratio of 3 (near the minimal optimal ratio) and lower concentrations of EtOH (40-69 %). Regarding the time variable, it is more difficult to establish a specific time for the best result since it varies greatly with the interaction of the other variables, although it is quite logic that the higher it is the better the results will be.

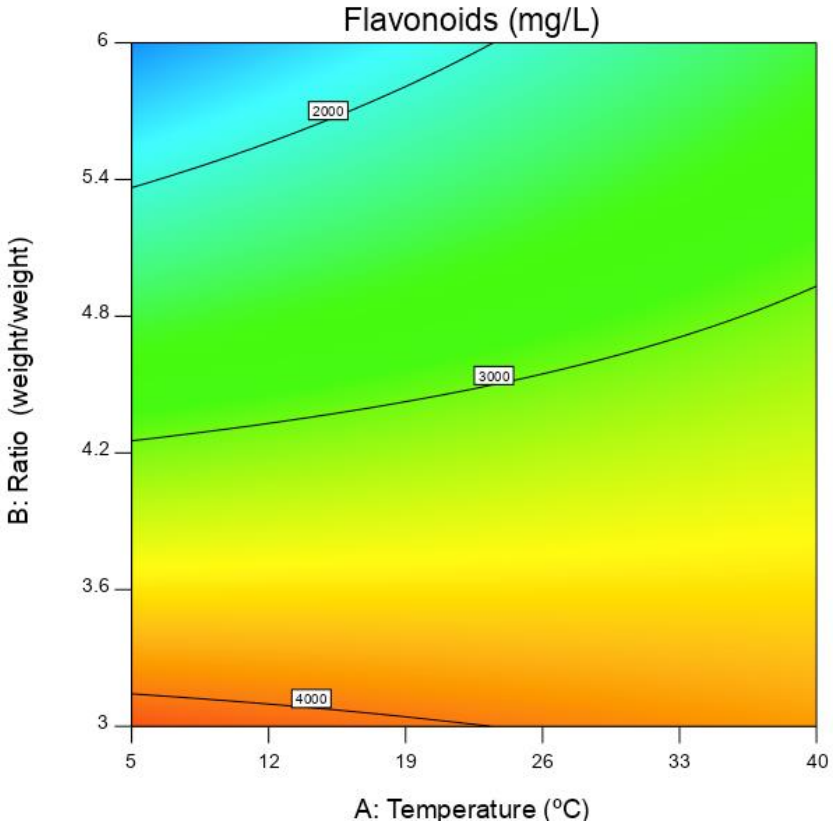


Figure 12. 2FI model graph of the model terms' interaction Temperature(A)·Ratio(B) from SDE (Flavonoids' concentration described by the lines).

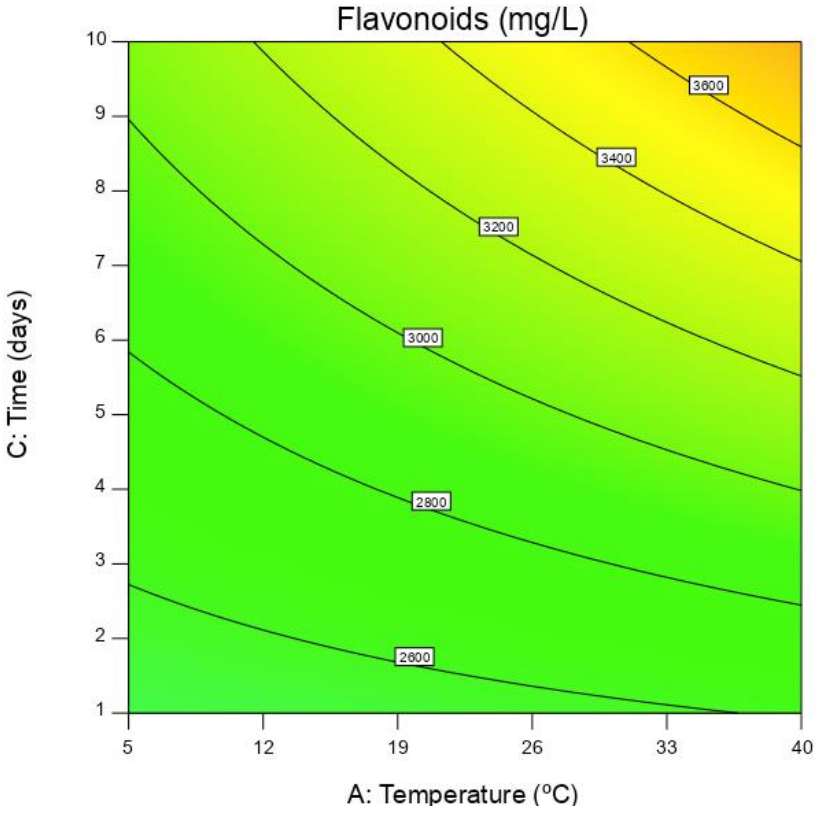


Figure 13. 2FI model graph of the model terms' interaction Temperature(A)·Time(C) from SDE (Flavonoids' concentration described by the lines).

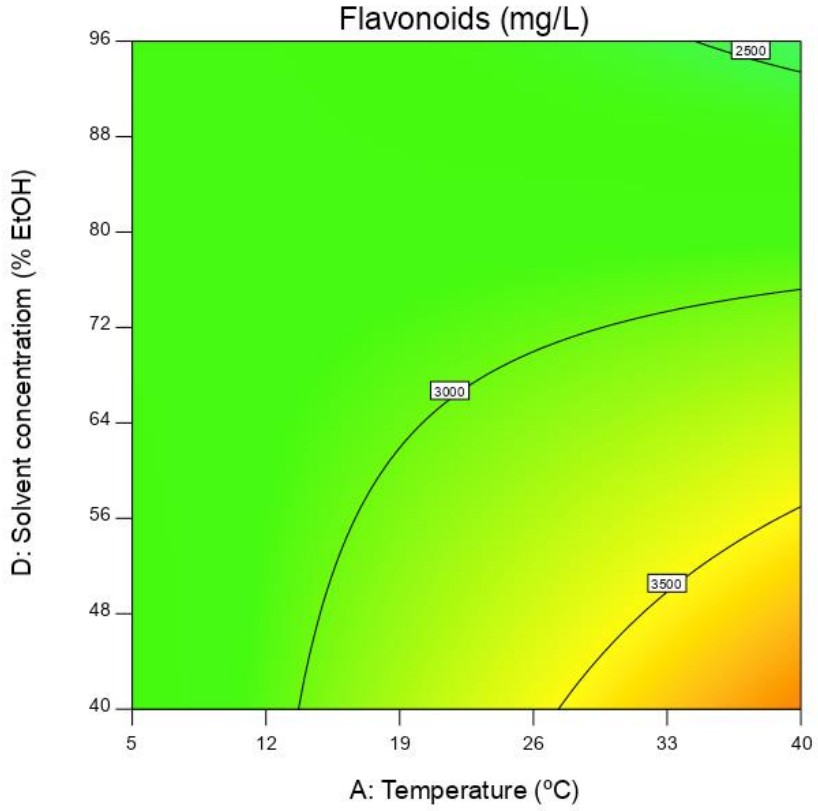


Figure 14. 2FI model graph of the model terms' interaction Temperature(A)·Solvent concentration(D) from SDE (Flavonoids' concentration described by the lines).

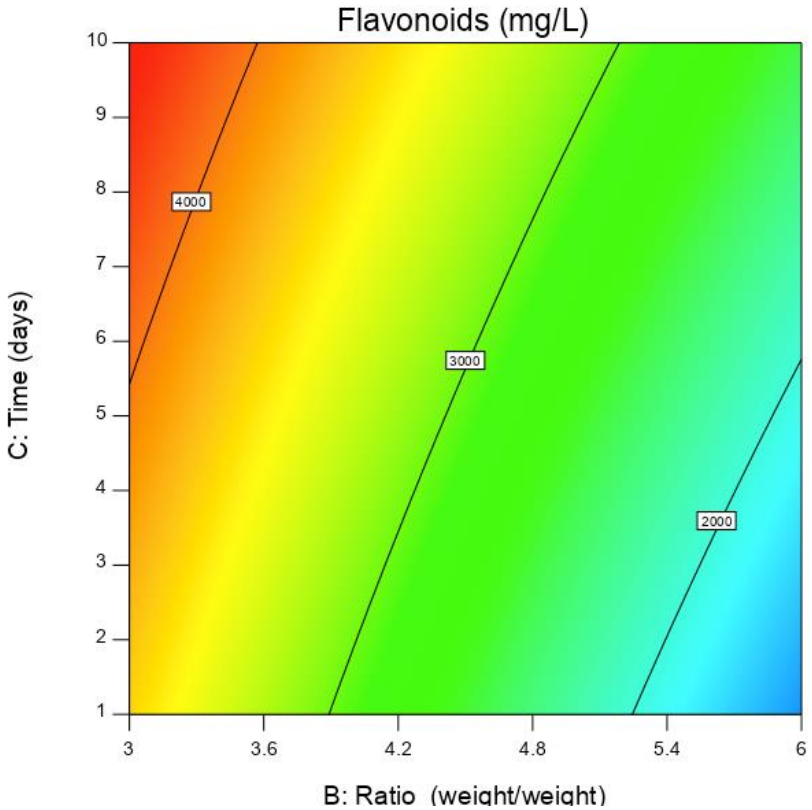


Figure 15. 2FI model graph of the model terms' interaction Ratio(B)·Time(C) from SDE (Flavonoids' concentration described by the lines).

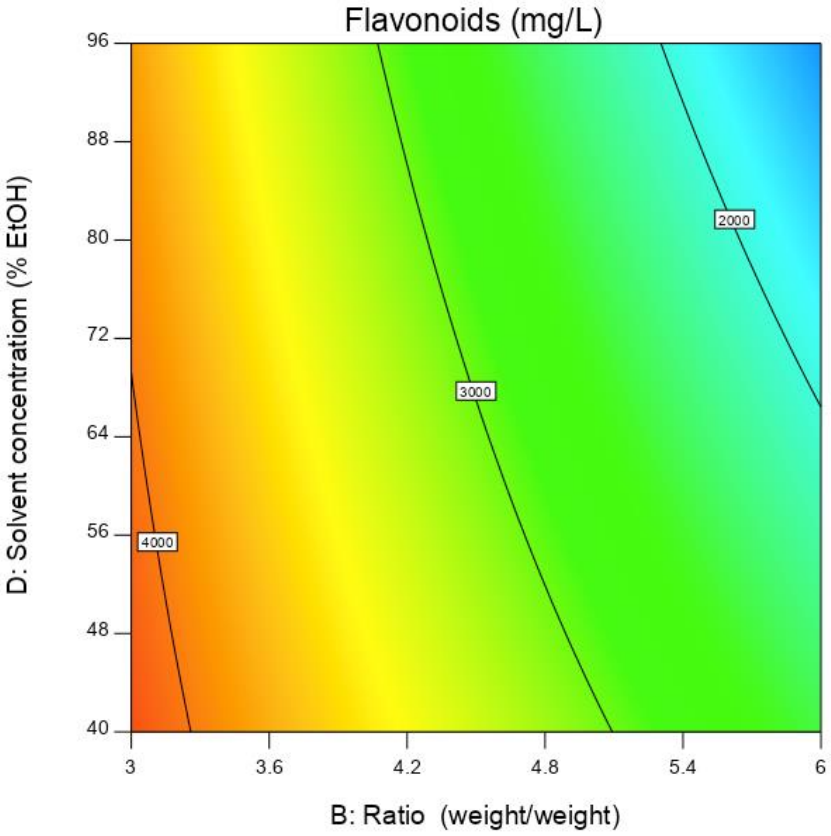


Figure 16. 2FI model graph of the model terms' interaction Ratio(B)·Solvent concentration(D) from SDE (Flavonoids' concentration described by the lines).

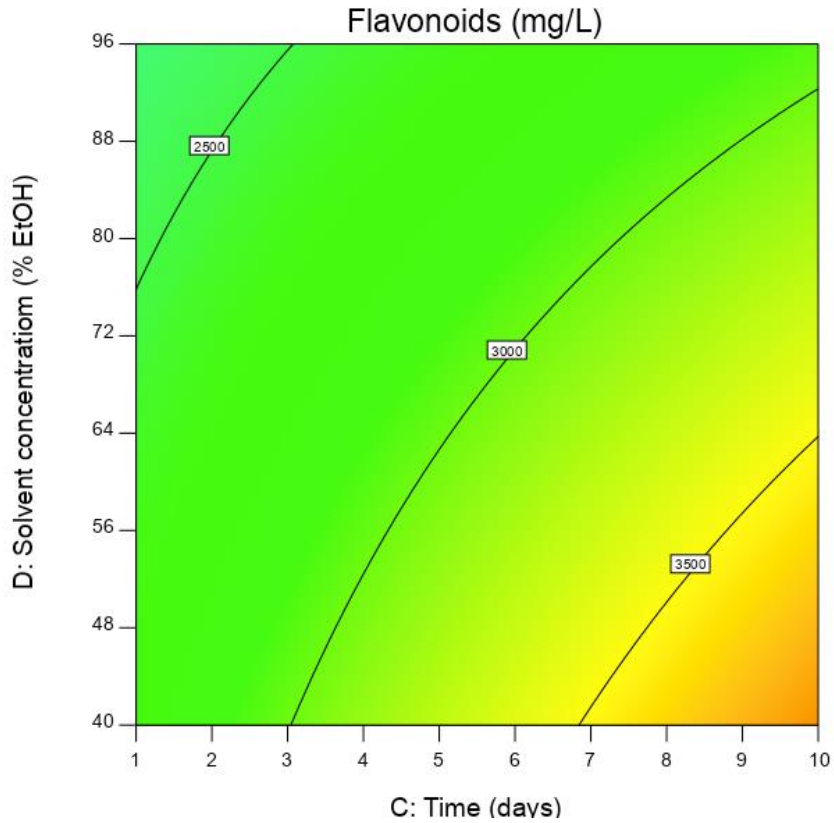


Figure 17. 2FI model graph of the model terms' interaction Time(C)·Solvent concentration(D) from SDE (Flavonoids' concentration described by the lines).

Thus, with all these data, the software Design Expert also provided a prediction of the optimized extraction conditions by the generated model, since the peak of flavonoids extraction was not reached in this experiment as shown in Figure 18. Additionally, the operator has the option to give different levels of importance to the variables (being five is the most important), since the variables have different relevance in the extraction efficiency, as seen above in the analysis of the graphs. The selection of the variables' importance is described in Table 11.

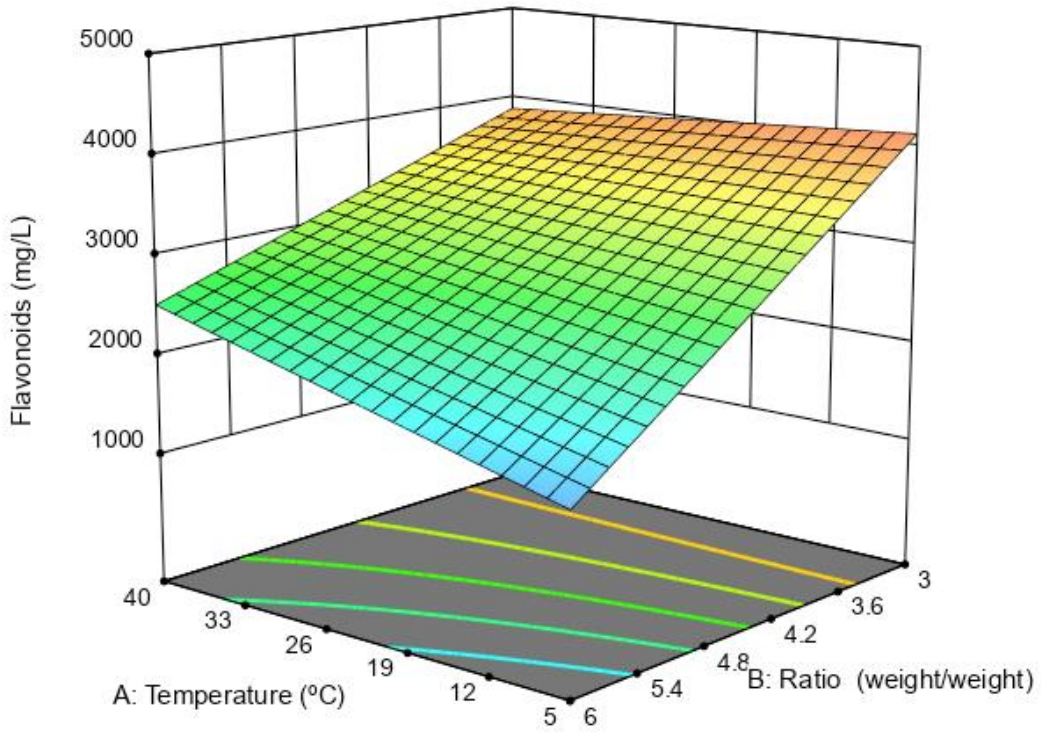


Figure 18. 3D model representation of the model terms' interaction Temperature(A)-Ratio(B) from SDE.

Table 11. Variables' importance in the optimization process of sea-buckthorn

| Name | Goal | Lower Limit | Upper Limit | Lower Weight | Upper Weight | Importance |
|---|---|---|---|---|---|---|
| **A: Temperature** | is in range | 5 | 40 | 1 | 1 | 4 |
| **B: Ratio** | is in range | 3 | 6 | 1 | 1 | 5 |
| **C: Time** | is in range | 1 | 10 | 1 | 1 | 2 |
| **D: Solvent concentration** | is in range | 40 | 96 | 1 | 1 | 4 |
| **Flavonoids** | maximize | 1030 | 4324 | 1 | 1 | 5 |

After the restrictions have been set, the software generated 100 solutions. The reason why the program generates all these solutions is because all of them are above the maximum value of 4324 mg/L of flavonoids. This could be an advantage for the optimization process because it can manipulate all the variables creating several alternative conditions in order to maximize the extraction. As an example, Table 12 shows some of these solutions. Nonetheless, it was still possible to find the best result in these 100 solutions, as it is the case of solution number 19. This solution was capable of extracting approximately 4940 mg/L of flavonoids, and the extraction conditions for such are: a temperature of 39.8 °C, a solvent ratio of 3.1, a solvent concentration of 41.9 % of EtOH and 9 days and half of extraction time. However, this is just a model extrapolation of the best result, which means that it is necessary to do an experimental validation of these results in order to confirm them; in other words, to validate the model.

Table 12. Exemplification of the solutions generated by the SDE

| Number | Temperature | Ratio | Time | Solvent concentration | Flavonoids | Desirability |
|---|---|---|---|---|---|---|
| **1** | 36.877 | 3.010 | 8.774 | 62.364 | 4364.182 | 1.000 |
| **2** | 8.060 | 3.010 | 8.975 | 88.307 | 4346.147 | 1.000 |
| **3** | 10.702 | 3.001 | 9.851 | 64.760 | 4326.336 | 1.000 |
| **4** | 38.019 | 4.536 | 9.978 | 48.522 | 4335.732 | 1.000 |
| **5** | 38.433 | 3.110 | 9.357 | 58.512 | 4491.024 | 1.000 |
| **6** | 23.106 | 3.087 | 8.801 | 52.480 | 4358.772 | 1.000 |
| **7** | 11.820 | 3.009 | 9.882 | 40.692 | 4346.678 | 1.000 |
| **8** | 5.487 | 3.015 | 7.618 | 83.818 | 4327.013 | 1.000 |
| **9** | 8.357 | 3.012 | 9.676 | 80.220 | 4336.536 | 1.000 |
| **10** | 5.158 | 3.103 | 8.777 | 93.767 | 4339.657 | 1.000 |
| **11** | 24.060 | 3.014 | 8.556 | 42.857 | 4483.596 | 1.000 |
| **12** | 35.072 | 4.075 | 9.713 | 49.345 | 4334.291 | 1.000 |
| **13** | 24.512 | 3.165 | 8.956 | 53.460 | 4340.867 | 1.000 |
| **14** | 39.813 | 5.658 | 9.913 | 40.404 | 4358.024 | 1.000 |
| **15** | 33.815 | 3.887 | 9.432 | 49.635 | 4324.153 | 1.000 |
| **16** | 19.783 | 3.042 | 9.738 | 50.847 | 4437.221 | 1.000 |
| **17** | 5.012 | 3.001 | 1.131 | 92.398 | 4324.027 | 1.000 |
| **18** | 38.083 | 3.340 | 6.603 | 44.573 | 4361.234 | 1.000 |
| **19** | 39.842 | 3.133 | 9.498 | 41.948 | 4939.614 | 1.000 |
| **20** | 7.762 | 3.002 | 9.842 | 68.680 | 4324.188 | 1.000 |
| **21** | 37.410 | 5.127 | 9.598 | 40.218 | 4328.180 | 1.000 |
| **22** | 5.137 | 3.009 | 6.048 | 87.014 | 4329.964 | 1.000 |
| **23** | 25.873 | 3.062 | 9.803 | 41.785 | 4657.329 | 1.000 |
| **24** | 16.662 | 3.036 | 8.951 | 46.928 | 4335.942 | 1.000 |
| **25** | 17.886 | 3.002 | 9.747 | 62.941 | 4358.216 | 1.000 |
| **26** | 25.480 | 3.107 | 9.287 | 55.810 | 4389.791 | 1.000 |
| **27** | 35.143 | 3.490 | 9.956 | 58.949 | 4358.418 | 1.000 |
| **28** | 16.547 | 3.066 | 9.576 | 46.987 | 4373.301 | 1.000 |
| **29** | 38.923 | 4.921 | 9.369 | 41.150 | 4396.307 | 1.000 |
| **30** | 11.807 | 3.004 | 9.872 | 56.187 | 4336.663 | 1.000 |
| **31** | 38.684 | 3.005 | 9.527 | 66.819 | 4353.165 | 1.000 |

# Chapter 7. Conclusions and Suggestions

## 7.1. Conclusions

Supplements are already a great part of people's lives, and their implementation is becoming increasingly diversified, with applications even more directed to the medical fields (nutraceuticals). Their interest is as well increasing every year, with consumers' special attention to the natural-based market.

Accordingly, the present work had the main focus of producing well characterized herbal extracts, for their application in supplements, specially from plants that are not extensively used in the present market. Additionally to this main purpose, the recognition of the best extraction conditions for the scale-up phase of the most interesting herbs, has also been defined as an objective of this study.

Firstly, all the selected plants were tested by their biological functionality, in three different types of solvent extraction. The results provided the information regarding which herbs and type of extraction reported the greatest potential of antioxidant inhibition, being the following herbs almost unanimously chosen in all extractions: silver birch; sea-buckthorn; rose hip; common hop; St. John's wort, all with more than 70 % of antioxidant inhibition.

Subsequently, the content of flavonoids' equivalents in the herbs was assessed. This test helped understanding if flavonoids would be the principal responsible for the biological effect at question, and in the same way to determine which extraction option is more prone to extract flavonoids. The study has showed that the 40 % EtOH and the 75 % glycerol extracts are more prone to extract flavonoids given the affinity of their polarity for the substances. Nevertheless, some of the herbs have been shown to be excellent sources of flavonoids, but without an important

relevance in antioxidant potential, and therefore more suitable for other applications, such as the cosmetic market.

The correlation of the dry weight data with the flavonoids results played a crucial role on the characterization of the herbal extracts. This was transposed into percentage of flavonoids in the crude extracts of each plant for each solvent extraction option. The data helped to achieve a better understanding on the possibility of other functional groups being responsible (or not) for the antioxidant potential described.

Two herbs (common hop and sea-buckthorn) were selected to perform the optimization process of the extraction conditions. Resorting to the program Design Expert, two sets of experiments were designed, testing 4 variables (temperature, solvent ratio, solvent concentration and extraction time). Nonetheless, only the sea-buckthorn experiment was able to be accepted by a mathematical model, with the ideal extraction conditions of 39.8 °C, a solvent ratio of 3.1, 41.9 % of EtOH and 9 days and half of extraction time.

## 7.2. Suggestions

The present work provided a better understanding on which extraction conditions maximized the extraction of flavonoids and which relations these substances had with the biological effect at question, on each plant and solvent extractor. On this perspective, it would be interesting to apply the same efforts to the study of other functional groups that also have significant interest for this same application (e.g., polyphenolic compounds). Additionally, the study of other biological activities, already mentioned (e.g., anti-inflammatory and the cytotoxic activities), should be performed

Considering the several applications of these extracts in the food industry, it should be interesting to test their application in smart food packing as oxidation inhibitors, and flavour and odours absorbers or releasers.

The reduction of the model for the optimization process could improve the accuracy of the mathematical representation of the data. Besides this, it would be also of great importance to do the experimental validation of the mathematical model by obtaining the expected concentration of flavonoids for the optimized conditions.

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# Appendix

## Appendix A – Vitamin C's calibration curve per absorbency and inhibition (%)

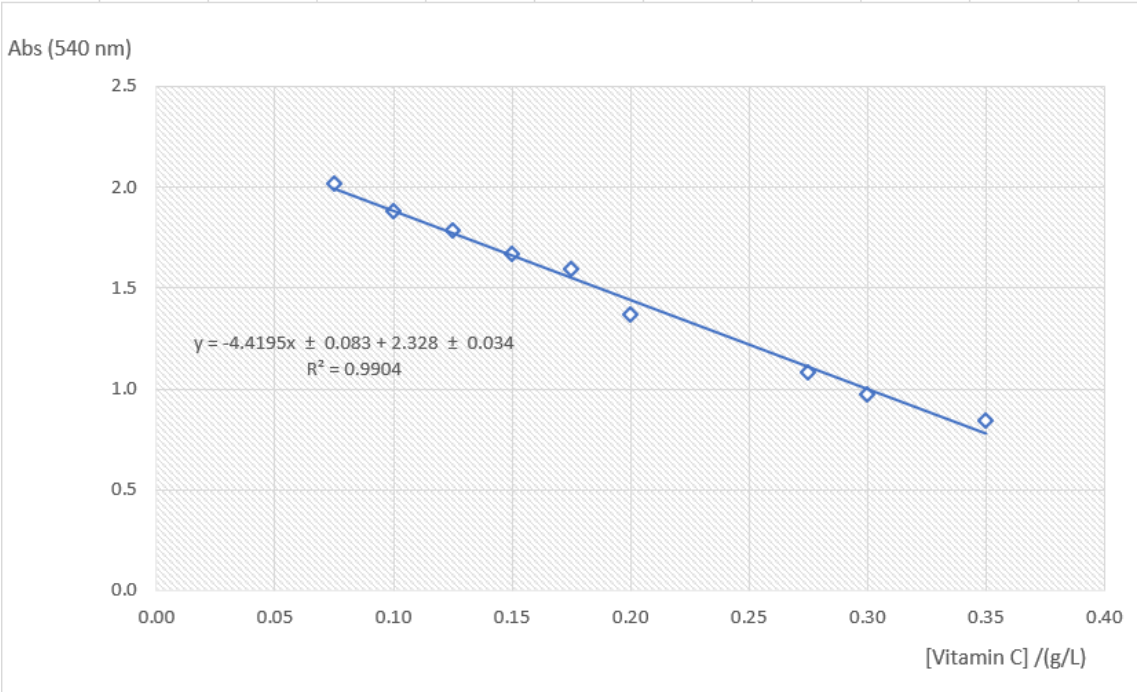


**Figure A. 1** Vitamin C' calibration curve per absorbency

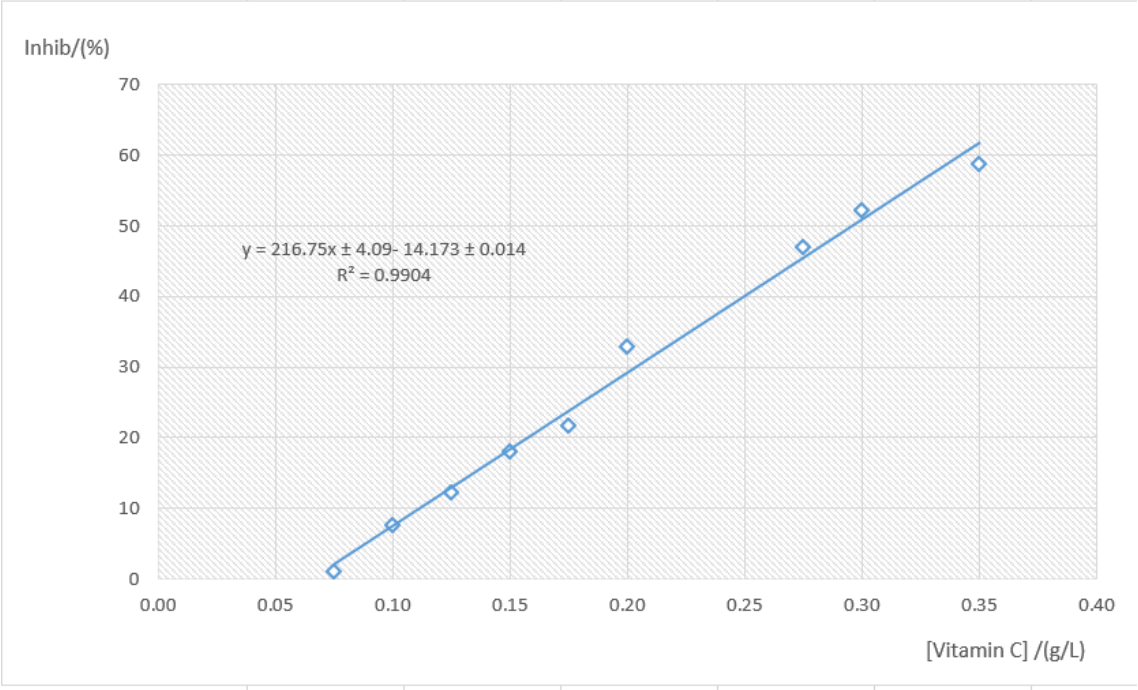


**Figure A. 2** Vitamin C' calibration curve per inhibition percentage

# Appendix B – Rutin's calibration curve

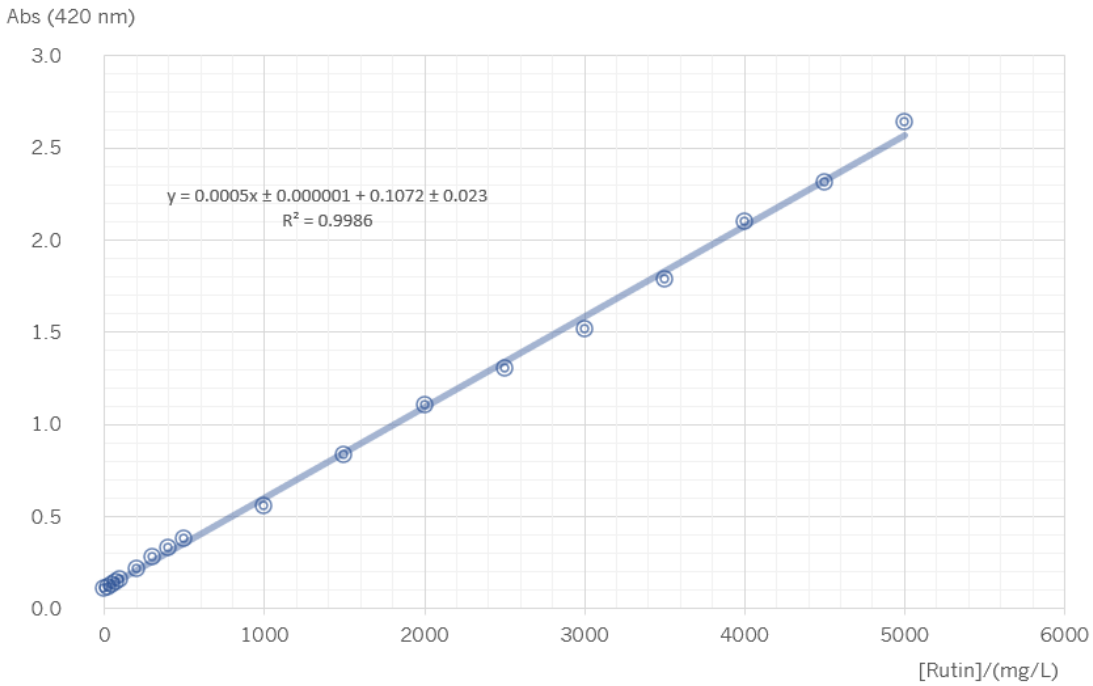


**Figure B. 1** Rutin's calibration curve

## Appendix C – DPPH radical inhibition results of the herbs

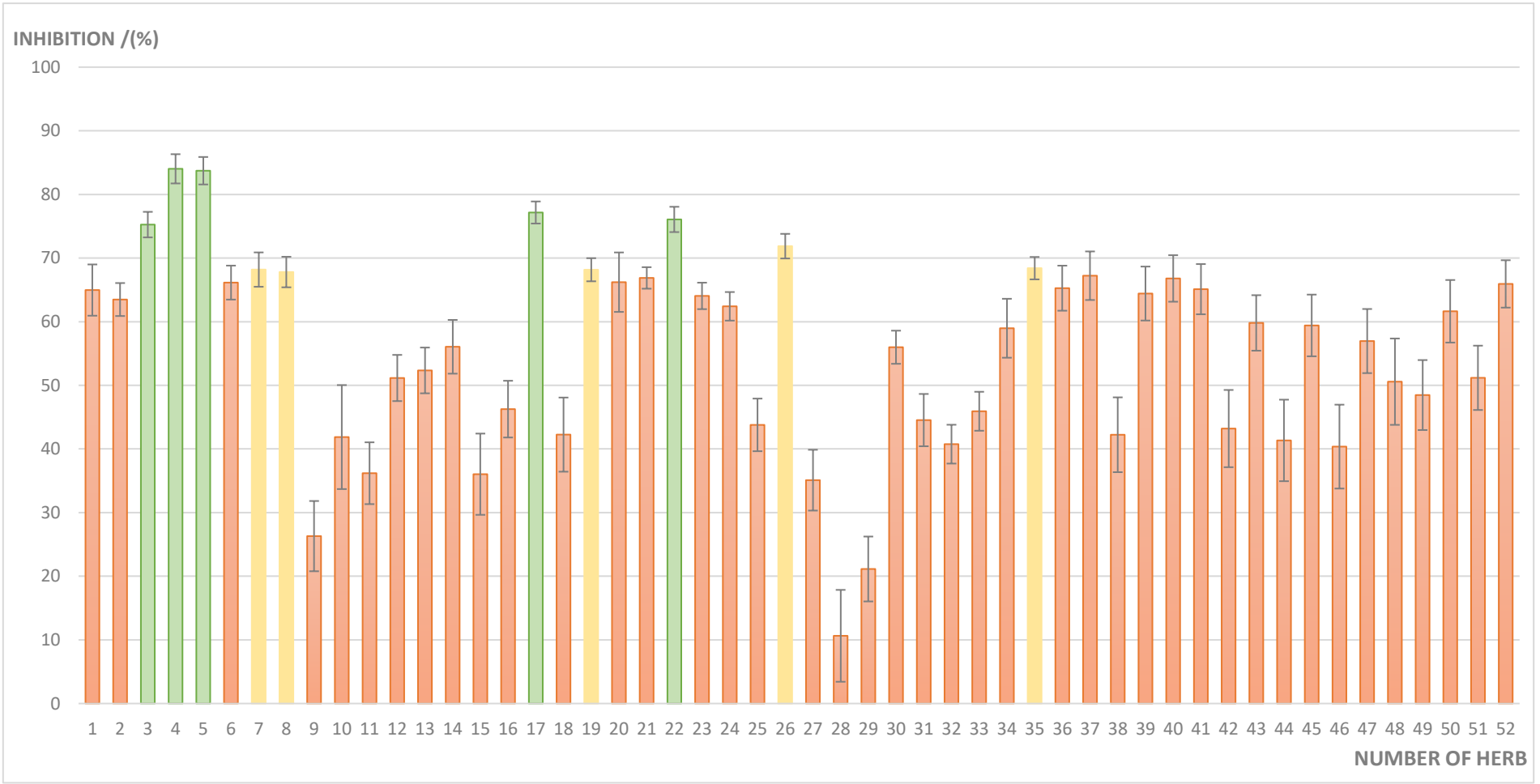


**Figure C. 1** DPPH radical inhibition results of the E40 extracts

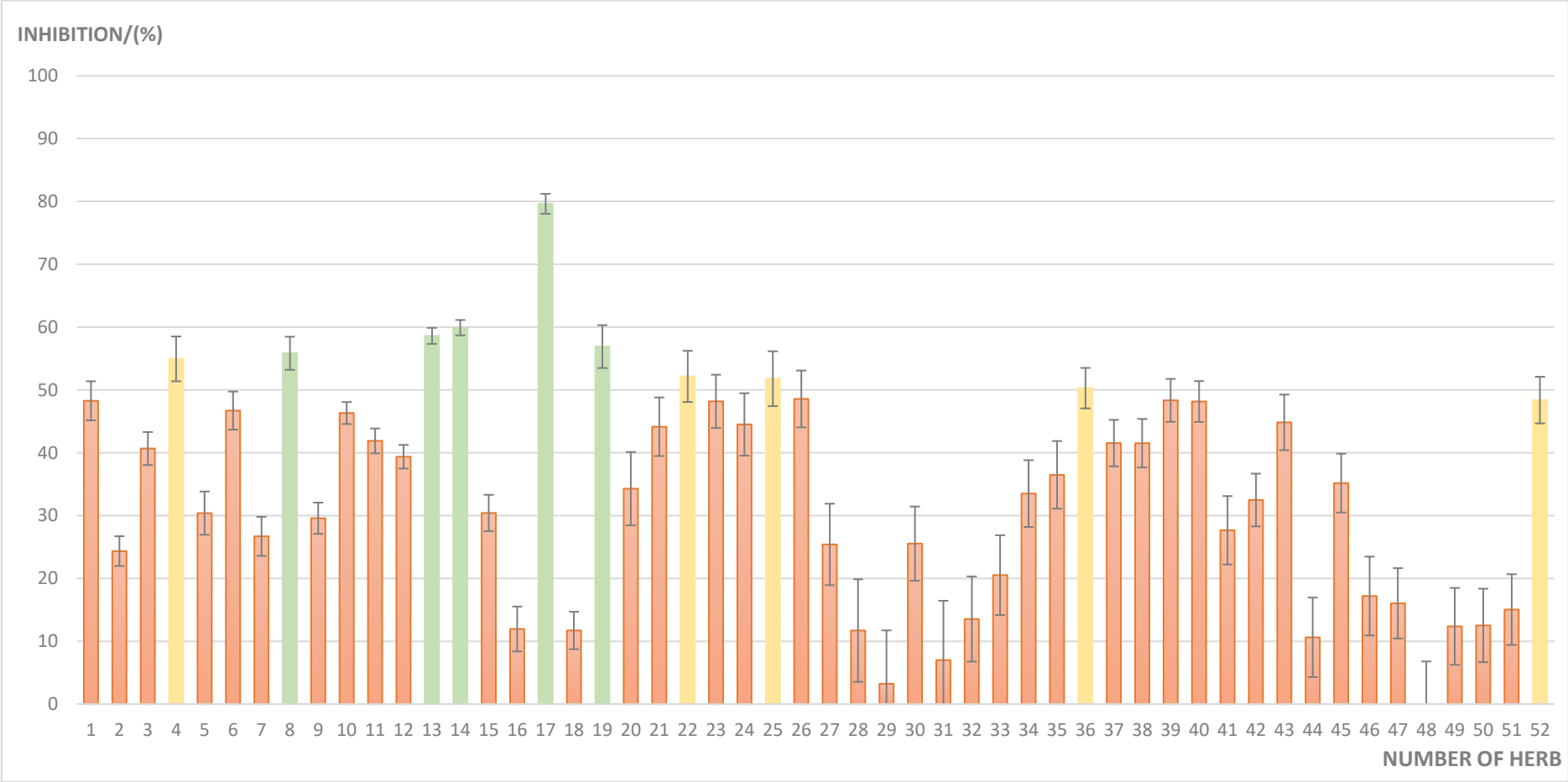


**Figure C. 2** DPPH radical inhibition results of the E96 extracts

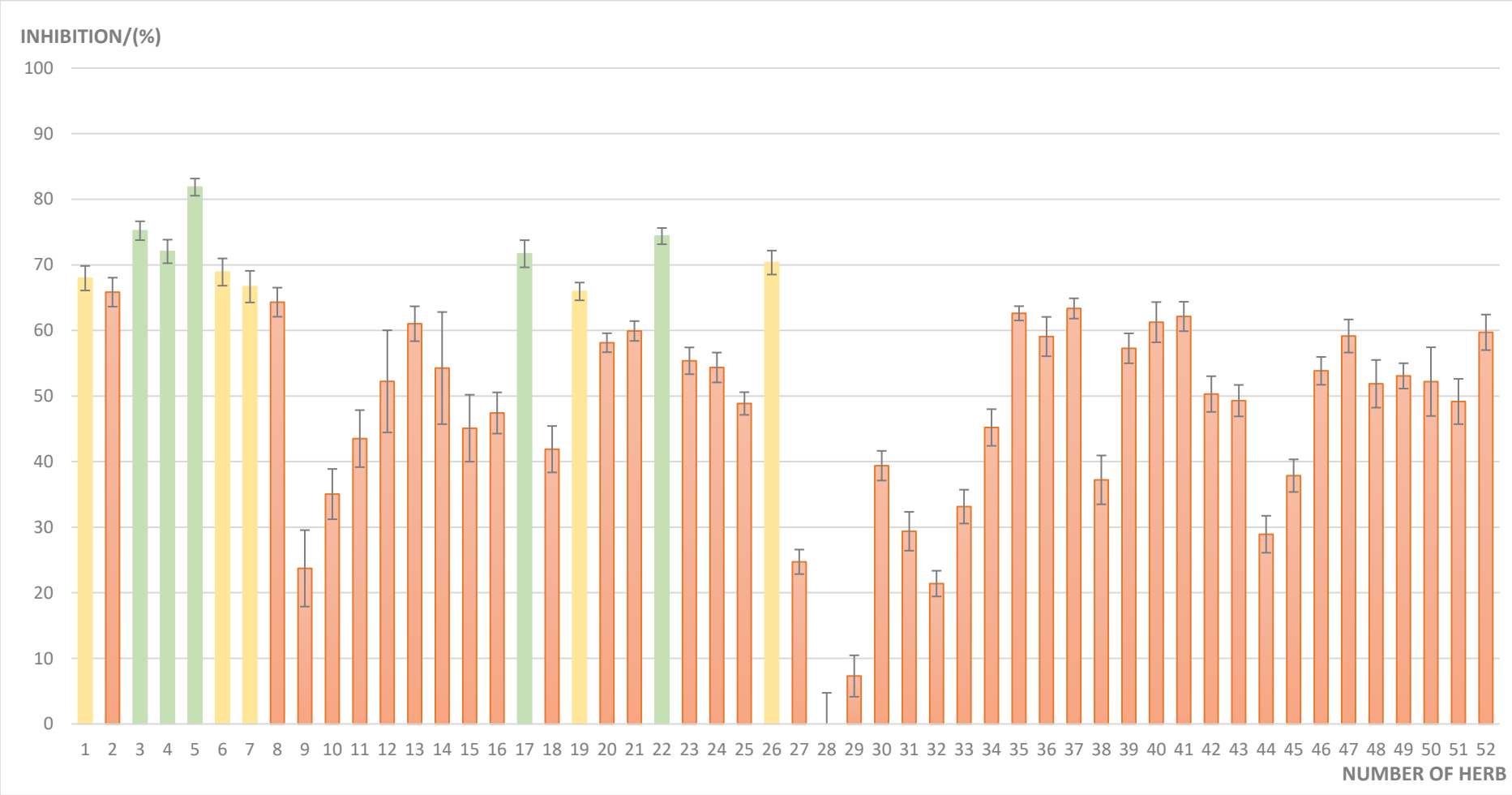


**Figure C. 3** DPPH radical inhibition results of the G75 extracts

## Appendix D – Flavonoids' quantification results of the herbs

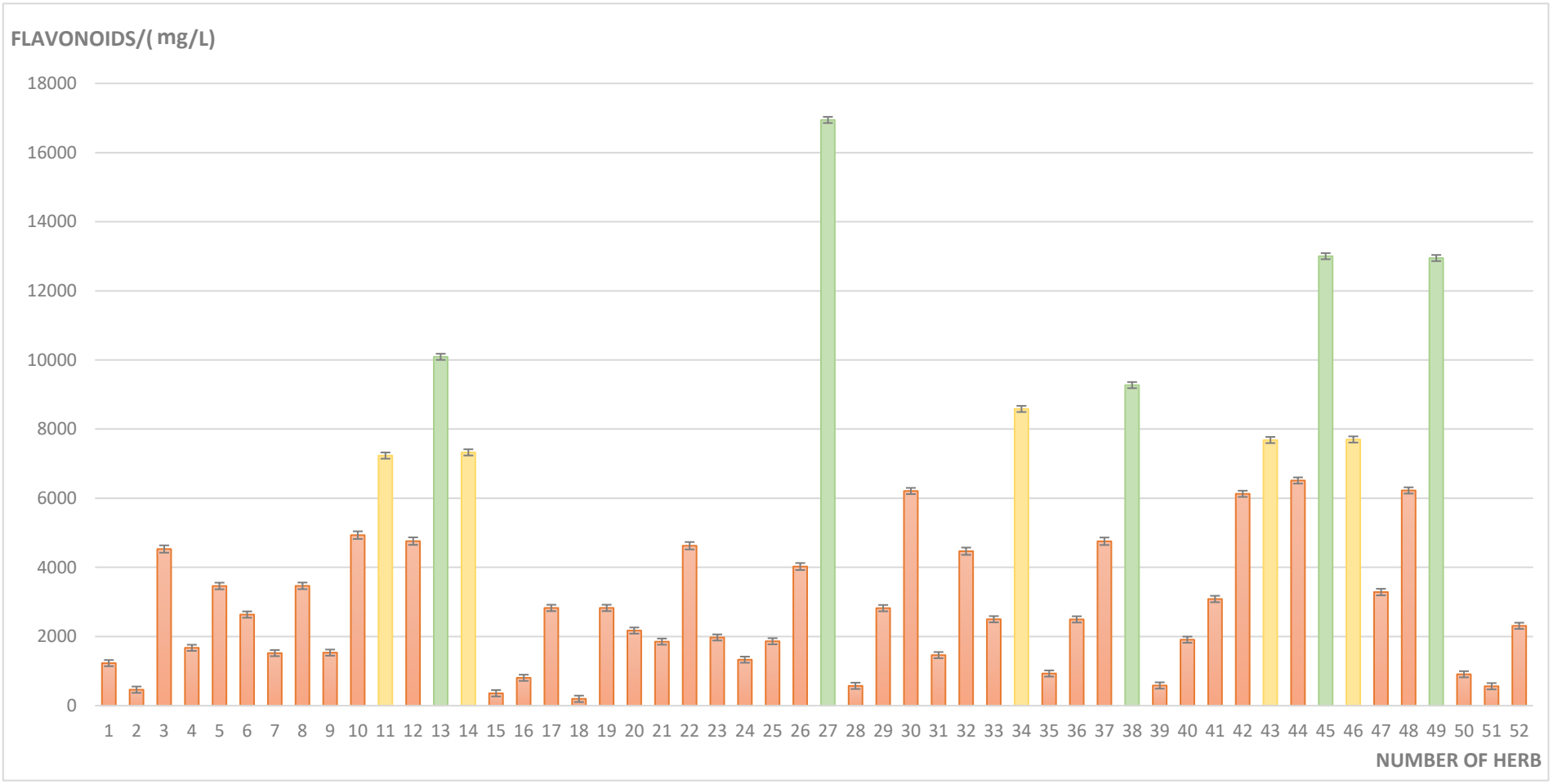


**Figure D. 1** Flavonoids' quantification results of the E40 extracts

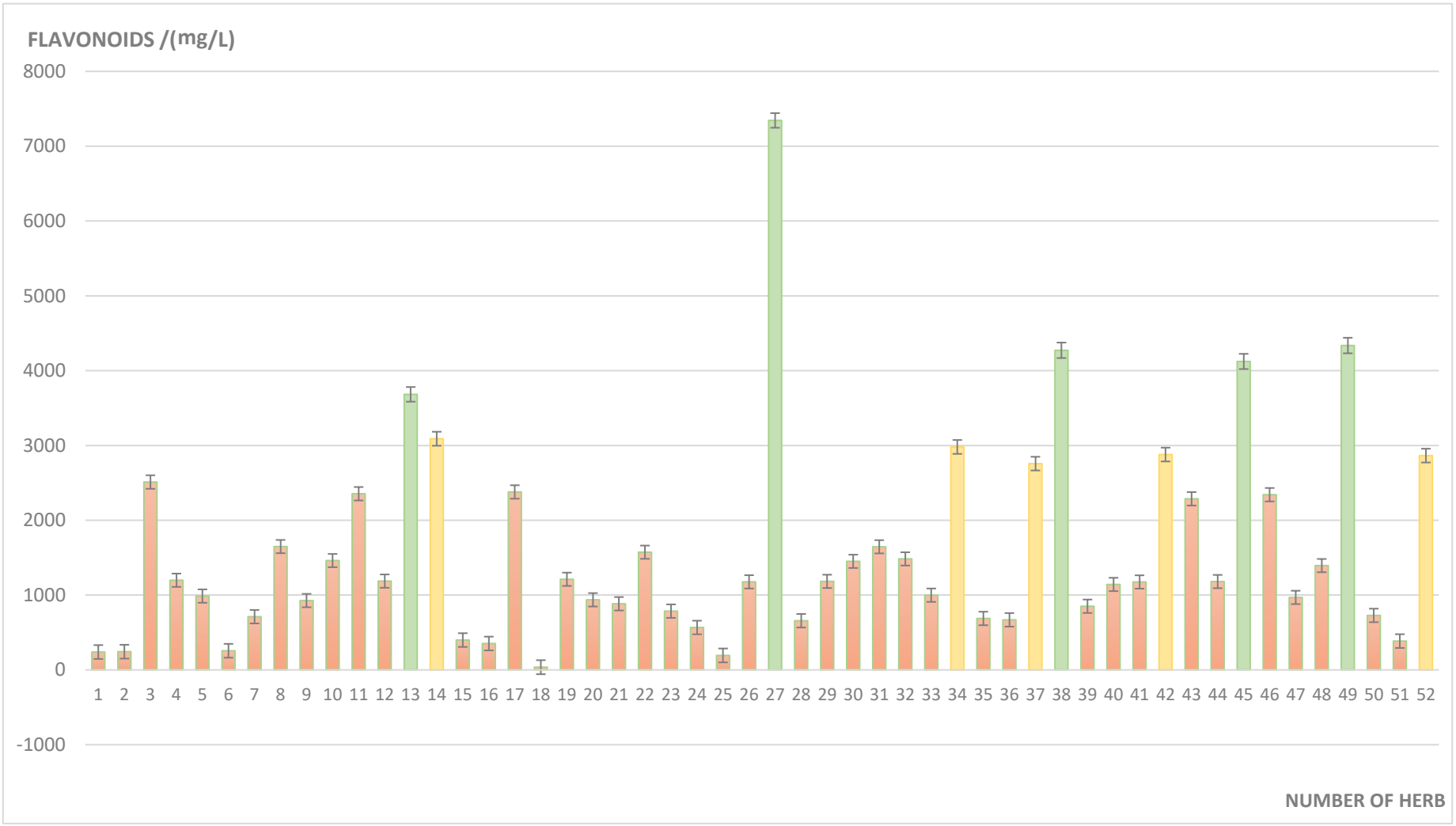


**Figure D. 2** Flavonoids' quantification results of the E96 extracts

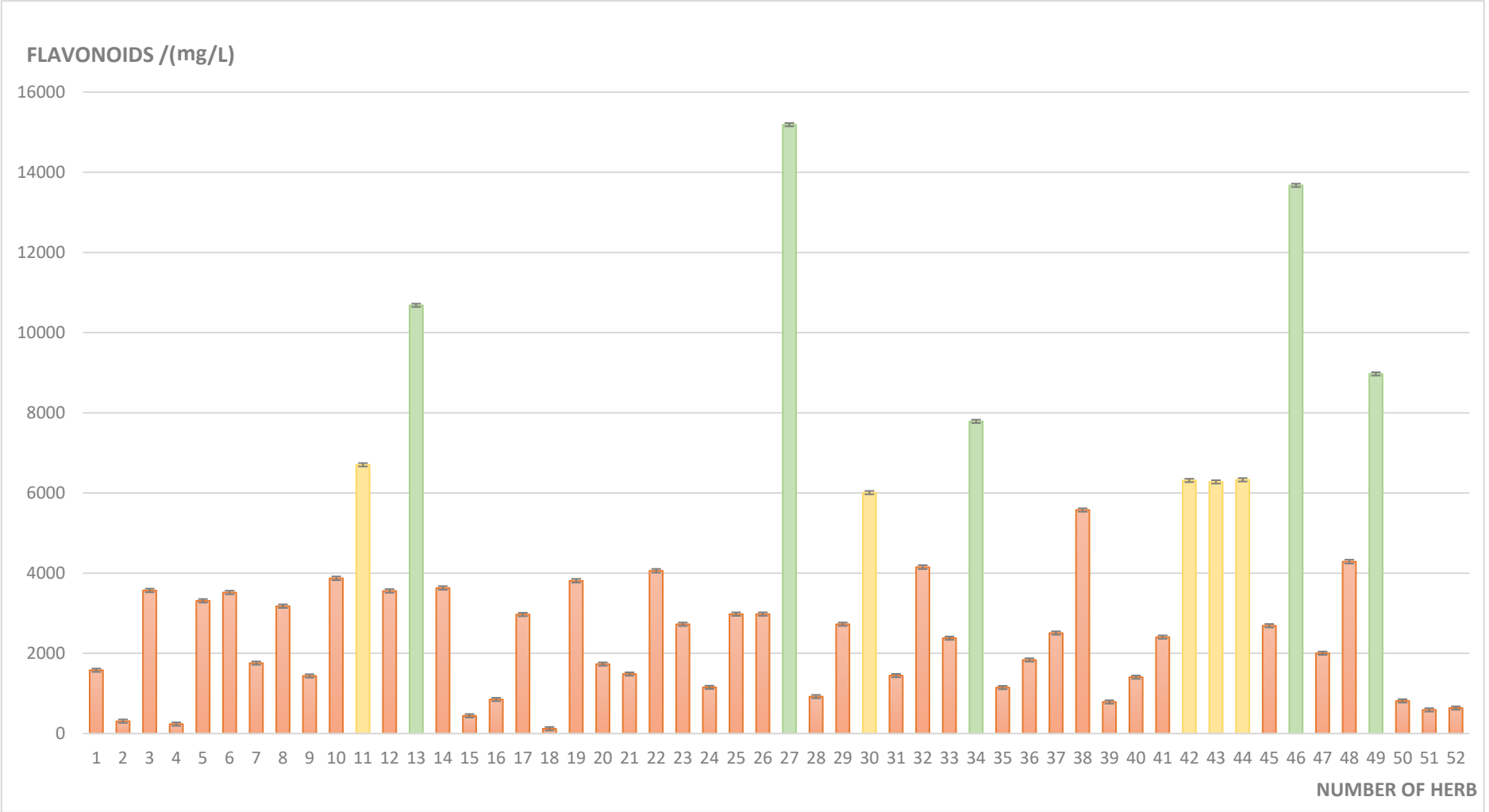


**Figure D. 3** Flavonoids' quantification results of the G75 extracts

# Appendix E – Experimental results of the optimization process

**Table E. 1** Experimental results of common hop

| Run | Temperature/(ºC) | Ratio/(w/w) | Time/(days) | % EtOH (w/w) | Flavonoids/(mg/L) |
|---|---|---|---|---|---|
| **1** | 22 | 9 | 10 | 70 | 2167 |
| **2** | 40 | 6 | 1 | 96 | 1912 |
| **3** | 5 | 6 | 10 | 40 | 2341 |
| **4** | 40 | 6 | 10 | 96 | 2270 |
| **5** | 40 | 9 | 6 | 70 | 2251 |
| **6** | 22 | 9 | 6 | 96 | 1500 |
| **7** | 40 | 12 | 10 | 40 | 2072 |
| **8** | 22 | 6 | 6 | 70 | 3167 |
| **9** | 22 | 9 | 6 | 70 | 2148 |
| **10** | 22 | 9 | 6 | 70 | 2221 |
| **11** | 22 | 9 | 1 | 70 | 1611 |
| **12** | 40 | 12 | 1 | 40 | 2004 |
| **13** | 22 | 12 | 6 | 70 | 1823 |
| **14** | 5 | 9 | 6 | 70 | 2324 |
| **15** | 5 | 12 | 1 | 96 | 858 |
| **16** | 22 | 9 | 6 | 70 | 2118 |
| **17** | 5 | 12 | 10 | 96 | 1291 |
| **18** | 22 | 9 | 6 | 70 | 2178 |
| **19** | 22 | 9 | 6 | 40 | 2609 |
| **20** | 5 | 6 | 1 | 40 | 2197 |

**Table E.2** Experimental results of sea-buckthorn

| Run | Temperature/(ºC) | Ratio/(w/w) | Time/(days) | % EtOH (w/w) | Flavonoids/(mg/L) |
|---|---|---|---|---|---|
| **1** | 22 | 4.5 | 10 | 70 | 3055 |
| **2** | 40 | 3 | 1 | 96 | 2903 |
| **3** | 5 | 3 | 10 | 40 | 4201 |
| **4** | 40 | 3 | 10 | 96 | 3677 |
| **5** | 40 | 4.5 | 6 | 70 | 3349 |
| **6** | 22 | 4.5 | 6 | 96 | 2586 |
| **7** | 40 | 6 | 10 | 40 | 4324 |
| **8** | 22 | 3 | 6 | 70 | 4039 |
| **9** | 22 | 4.5 | 6 | 70 | 2871 |
| **10** | 22 | 4.5 | 6 | 70 | 3292 |
| **11** | 22 | 4.5 | 1 | 70 | 2605 |
| **12** | 40 | 6 | 1 | 40 | 2587 |
| **13** | 22 | 6 | 6 | 70 | 1975 |
| **14** | 5 | 4.5 | 6 | 70 | 2961 |
| **15** | 5 | 6 | 1 | 96 | 1030 |
| **16** | 22 | 4.5 | 6 | 70 | 3062 |
| **17** | 5 | 6 | 10 | 96 | 1573 |
| **18** | 22 | 4.5 | 6 | 70 | 3142 |
| **19** | 22 | 4.5 | 6 | 40 | 3293 |
| **20** | 5 | 3 | 1 | 40 | 3412 |

# Appendix F – Dry Weight Results of the alcoholic extracts

**Table F.1** Dry weight results for each alcoholic extracts

| No. | Plant Name | Dry Weight /(g/L) E40* | Dry Weight /(g/L) E96* |
|---|---|---|---|
| **1** | Dried burdock | 25.71 | 4.42 |
| **2** | Couch grass | 13.95 | 4.36 |
| **3** | Silver birch | 17.99 | 12.33 |
| **4** | Sea-buckthorn | 28.23 | 17.56 |
| **5** | Rose hip | 27.40 | 6.47 |
| **6** | Red clover | 14.05 | 4.5 |
| **7** | Common mallow | 23.94 | 5.58 |
| **8** | Elderberry | 29.73 | 9.06 |
| **9** | Galega/ Goat's-rue | 17.18 | 4.49 |
| **10** | European Goldenrod | 22.18 | 5.19 |
| **11** | Hawthorn | 20.45 | 7.26 |
| **12** | Yarrow | 18.23 | 4.43 |
| **13** | Wild Strawberry | 26.68 | 10.39 |
| **14** | Heather | 17.15 | 9.06 |
| **15** | Dandelion | 29.36 | 3.28 |
| **16** | Common Soapwort | 42.27 | 4.33 |
| **17** | Common hop | 14.32 | 14.76 |
| **18** | Garlic | 67.30 | 0.48 |
| **19** | Small-leaved lime | 15.97 | 3.4 |
| **20** | Yellow sweet clover | 17.87 | 4.55 |
| **21** | Wormwood | 17.93 | 6.04 |
| **22** | St John's-wort | 15.73 | 5.58 |
| **23** | Dandelion | 16.57 | 4.18 |
| **24** | Motherwort | 14.19 | 3.82 |
| **25** | Common comfrey | 43.72 | 1.82 |
| **26** | Daisy | 24.24 | 7.27 |

* The error for each concentration is of 0.07 g/L

**Table F.2** Dry weight results for each alcoholic extracts (continuation)

| No. | Plant Name | Dry Weight /(g/L) E40* | Dry Weight /(g/L) E96* |
|---|---|---|---|
| 27 | Lingonberry | 27.7 | 14.14 |
| 28 | Urtica | 12.98 | 3.41 |
| 29 | Hemp nettle | 23.39 | 5.13 |
| 30 | Basil | 18.43 | 4.97 |
| 31 | Ramson, Wild garlic | 23.67 | 8.85 |
| 32 | Veronica | 17.7 | 4.59 |
| 33 | Ground-ivy | 20.54 | 4.2 |
| 34 | Common Agrimony | 17.65 | 7.03 |
| 35 | Valerian | 22.92 | 7.16 |
| 36 | White horehound | 19.65 | 5.36 |
| 37 | Dyer's greenweed | 19.03 | 8.02 |
| 38 | Liquorice | 24.16 | 6.69 |
| 39 | Horsetail | 11.41 | 4.27 |
| 40 | Buckwheat | 15.23 | 4.15 |
| 41 | Calendula | 32.12 | 9.62 |
| 42 | Sage | 19.12 | 11.44 |
| 43 | Breckland thyme | 16.25 | 5.6 |
| 44 | Oregano | 16 | 3.69 |
| 45 | Willow | 18.38 | 7.58 |
| 46 | Rosemary | 15.73 | 11.65 |
| 47 | Chamomile | 28.23 | 6.56 |
| 48 | Elder flower | 24.74 | 5.68 |
| 49 | Wall germander | 29.22 | 10.01 |
| 50 | White Mustard | 12.84 | 8.27 |
| 51 | Common chicory | 20.05 | 6.44 |
| 52 | Milk thistle | 8.04 | 13.72 |

* The error for each concentration is of 0.07 g/L