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A contribution to the valorisation of a maritime plant: the *Corema album* (L.) D. Don

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Summary

Corema album is an endemic maritime plant of the Iberian Peninsula that can be found in sandy dunes along the Portuguese maritime coast. The shrub and its white berries, white crowberries, have long been known, being the berries traditionally harvested and consumed along the Atlantic littoral. However, although the berries are a source of nutrients and distinct from other berries because of their colour and acidic taste, they are not currently commercially exploited. The aim of this study was to contribute to the valorisation of *C. album* by determining the nutrient profile of its berries and addressing the phytochemical potential of the plant by quantification of total phenolic compounds content, in particular flavonoids, of extracts made with its berries and leaves. The plant showed to be a source of phenolic compounds, and the results suggest that *C. album* leaves extracts have a higher potential as a source of total phenolic compounds and flavonoids than the berries extracts.

Another aim of this study was to determine the phytochemical potential of the plant by measuring the antioxidant activity of extracts made with its berries and leaves. The extracts have antioxidant activity, being the antioxidant activity of the leaves extracts slightly superior.

The identification and quantification of phenolic compounds by HPLC was also made. From the results it was possible to verify that the berries extract contains trigonelline and the isomeric polyphenols 3-CQA, 4-CQA and 5-CQA and caffeic acid and the leaves extract also contains trigonelline and 3-CQA, 4-CQA

and 5-CQA, and contains ferulic acid. The results show that the berries extract is a better source of caffeic acid, chlorogenic acid and its isomers, 4-CQA and 5-CQA, and the leaves extract is a better source of trigonelline and ferulic acid.

With ATR-FTIR spectroscopy results was possible to identify major classes of compounds in the lyophilized berries powder and grinded leaves samples and, in the case of the berries sample, to recognize whether a particular peak or shoulder was due to the presence of seeds in the sample.

Another aim of this study was to determine the anticancer activity of extracts made with berries and leaves. The leaves extract showed to have a cytotoxic effect against the HT-29 cancer cell line, inducing cell death, visible by a reduction of cell viability through the MTT assay and also by a reduction of cell proliferation through the SRB assay.

Foods with the addition of white crowberries were also developed, cookies and bread, which were generally well accepted by the sensory panel of non-trained panelists to which they were submitted for sensory evaluation, showing that the development of new food products may be a way to make these berries more known to the public and start being commercialized.

Keywords: *Corema album*, *Corema album* berries, *Corema album* leaves, white crowberries, maritime plant, phytochemicals

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Resumo

A *Corema album* é uma planta marítima endémica da Península Ibérica que pode ser encontrada em zonas dunares ao longo da costa marítima portuguesa. O arbusto e a as suas bagas de cor branca, camarinhas, são há muito conhecidos, sendo as bagas tradicionalmente colhidas e consumidas ao longo do litoral atlântico. No entanto, apesar de as bagas serem uma fonte de nutrientes e distintas das outras bagas devido à sua cor e gosto ácido, não são actualmente exploradas comercialmente. O objectivo deste estudo foi contribuir para a valorização da *C. album* através da determinação do perfil nutricional das suas bagas e do potencial em fitoquímicos pela quantificação do conteúdo total em compostos fenólicos, em particular de flavonóides, de extractos das suas bagas e folhas. A planta mostrou ser uma fonte de compostos fenólicos, e os resultados sugerem que os extractos das folhas de *C. album* têm maior potencial como fontes de compostos fenólicos totais e flavonóides do que os extractos das bagas.

Outro objectivo deste estudo foi determinar o potencial fitoquímico da planta pela medição da actividade antioxidante de extractos das suas bagas e folhas. Os extractos possuem actividade antioxidante, sendo a actividade antioxidante dos extractos de folhas ligeiramente superior.

Foi também realizada a identificação e quantificação de compostos fenólicos por HPLC. Pelos resultados foi possível verificar que o extracto de bagas contem trigonelina e as formas isoméricas dos polifenóis 3-ACQ, 4-ACQ e 5-ACQ e ácido cafeico e o extracto de folhas também contem trigonelina e 3-ACQ, 4-ACQ

e 5-ACQ, e contem ácido ferúlico. Os resultados mostraram que o extracto de bagas é uma melhor fonte de ácido cafeico, ácido clorogénico e os seus isómeros, 4-ACQ e 5-ACQ, e o extracto de folhas é uma melhor fonte de trigonelina e ácido ferúlico.

Com os resultados da espectroscopia por ATR-FTIR foi possível identificar as principais classes de compostos presentes nas amostras de pó de bagas liofilizadas e de folhas trituradas e, no caso da amostra de bagas, perceber se um determinado pico ou ombro estava presente devido à presença de sementes na amostra.

Outro objectivo deste estudo foi determinar a actividade anticancerígena dos extractos das bagas e folhas. O extracto de folhas mostrou ter um efeito citotóxico contra a linha celular cancerígena HT-29, induzindo a morte celular, o que foi visível pela redução da viabilidade celular através do ensaio de MTT e também pela redução da proliferação celular através do ensaio de SRB.

Foram também desenvolvidos alimentos com a adição de camarinhas, bolachas e pão, que foram globalmente bem aceites pelo painel de provadores não treinados a que foram submetidos para análise sensorial, mostrando que o desenvolvimento de novos produtos alimentares pode ser uma forma de dar a conhecer estas bagas ao público e de iniciar a sua comercialização.

Palavras-chave: *Corema album*, bagas de *Corema album*, folhas de *Corema album*, camarinhas, planta marítima, fitoquímicos

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1. Introduction

1.1. *Corema album* general description

Corema album (L.) D. Don is a plant whose fruits, "white crowberries" or "camarinhas" in Portuguese, have been eaten by people in the Iberian Peninsula since at least the Early Neolithic. They were traditionally consumed along the Atlantic littoral of the Iberian Peninsula and have been used in popular medicine as antipyretic to treat fevers and as a vermifuge against pinworm infections[1,2]. The plant was also used to make rustic brushes, which may explain the origin of the genus name *Corema* that came from the Greek verb "korema" which means "broom". Although the plant and its fruits have been known and consumed since a long time, the berries never became a commercial crop. However, the shrub has been cultivated has an ornamental plant, and the berries are reported to be sold fresh in a few public markets in Galicia, Spain. Local populations that are familiar with the berries continue to harvest and consume it nowadays[3,4]. Current archaeobotanical research is expanding the record on the importance of this plant in human culture, dating back as far as the Early Neolithic[2]. *C. album* belongs to the division Spermatophyta, subdivision Magnoliophytina (Angiospermae), class Magnoliopsida, subclass Asteridae and order Ericales, being a member from the

Ericaceae family[5]. Two subspecies exist: subsp. *album* and subsp. *azoricum*. *C. album* subsp. a*lbum* occurs on the Atlantic coast of the Iberian Peninsula and subsp *Azoricum* in the Portuguese archipelago of Azores[6]. It has 26 chromosomes[3].

C. album is an endemic maritime plant of the Iberian Peninsula that can be found in coastal areas, particularly in sand dunes but also at rocky sites[6]. In Portugal this plant can be found on land by the sea, from north to south of the country[7] (Figure 1). The plant consists of a dioecious and perennial shrub which can reach 1 m and with numerous branches. The leaves are sub-verticillated and glabrous and are distributed along the branches in whorls of three or four, with short petioles which tend to lie against the stem. The roots are thick and spreading. The flowers are small with pink petals[8] and the fruits are small, round and usually white and can acquire a pink colour[3]. They have a strong skin and usually three large seeds with a thick endocarp. The berries grow during late spring and early summer[3,6] and have a distinct fresh and acidic taste[2].

1.2. Plant potential

The white crowberries have a different and distinct colour from the currently commercialized fruits, and also from the other berries present in the markets. Its acidic taste and flavour are also very distinct in relation to the sensorial characteristics of other berries. Due to these characteristics they have the potential to join the other edible berries already so well known to us and becoming one more source of nutrients. In addition to the current distribution, the plant also has potential to be distributed through other locations, as shown in Figure 1[2].

Recently, the berries were described as a good source of phenolic acids and flavonols[9]. Their characteristics and health benefits can be better known by carrying out further studies. The development of new food products with these berries is an interesting way to make them more known to the public and to start being marketed.

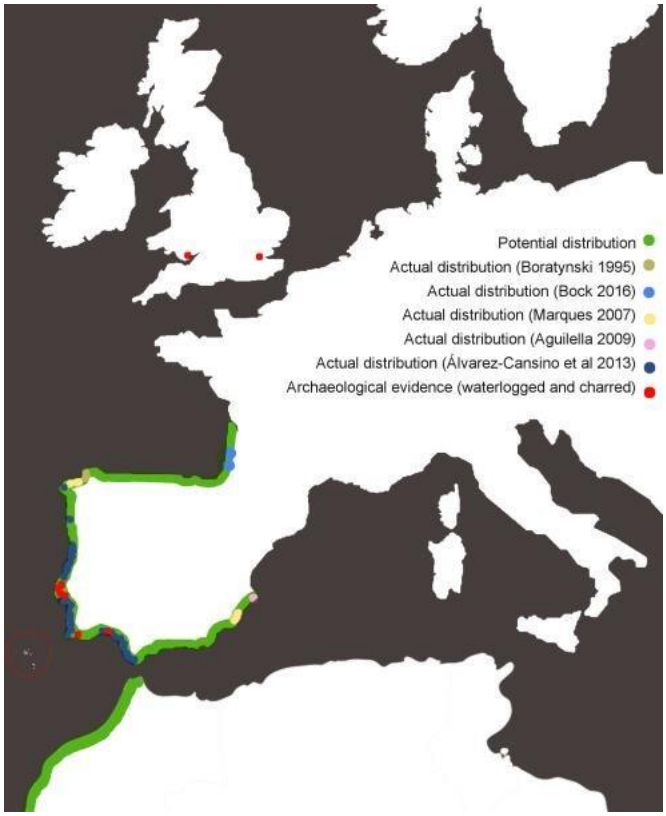

Figure 1 – Current and potential distributions of *C. album* with plotted archaeological records. The potential distribution is a minimum estimate based on the current distribution areas recorded in most references or successfully introduced and naturalized populations outside the frequently cited range (from [2]).

1.3. Phytochemicals and their importance to human health

Phytochemicals constitute a diverse group of compounds present in plants, being part of their secondary metabolism. They have evolved as a response to the interaction between the plant and the environment and provide varying levels of protection to biotic and abiotic stress conditions. The number of phytochemicals has been estimated in the hundreds of thousands. Their biosynthesis is tightly spatio-temporally regulated and often restricted to specialized cells[10].

Phytochemicals are not recognised as essential components in the human diet since a lack in their consumption is not associated with a specific deficiency[11]. These compounds are present in the human diet through fruits and vegetables and have numerous functions at the biological level, having health promoting effects[12]. In recent years the interest in these compounds has been increasing since

their consumption has been associated with a reduction in the risk of occurrence of numerous diseases: cancer[13,14], cardiovascular diseases[15], diabetes[16], osteoporosis[17,18], cataracts[19] and cognitive impairment[20,21] are examples[22]. These effects are associated to the antioxidant actions of phytochemicals and their capacity to generate signals that promote the cellular defence system[23].

Indeed, some phytochemicals have been used therapeutically since ancient times. The discovery of the phytochemicals responsible for the biological effect and their molecular structures are the basis of many modern pharmaceuticals. Some examples are artemisinin from the plant *Artemisia annua*, very effective treatment of malaria, including highly drug-resistant strains[24]; paclitaxel (with the commercial name taxol), an anti-cancer drug originally extracted from the Pacific Yew tree (*Taxus brevifolia*)[25] and reserpine from the plant *Rauwolfia serpentine*, effective in the treatment of hypertension[26]. Knowing the dose required for the desired effect is essential for the success of phytochemicals and for avoiding side effects.

Primary and secondary plant metabolites have different functions. The primary plant substances are proteins, fats, carbohydrates and fibres which contribute to energy metabolism and to the structure of the plant cell. The secondary plant metabolites are non-nutritive and present in very small amounts, but essential in the interaction of the plant with the surrounding environment[12]. They serve as defence and increase the plant tolerance to herbivore, insect and pathogen attacks. Others participate in shielding plants from unfavourable environmental conditions, such as high UV or drought. Particular types of phytochemicals are also involved in plant development and growth, being integral components of the plant signalling machinery that can directly serve as signalling molecules or interfere with the signalling activity of other molecules[10].

Phytochemicals can be classified into large classes, based on the starting point of their biosynthesis: phenolic compounds, carotenoids, products with nitrogen, alkaloids and organosulfur compounds (Figure 2)[27]. Every class of phytochemicals is further divided in many other smaller subclasses forming a complex diagram of classification, with a wide range of isomeric forms and different substituents, that show the different biological active effect[10,27].

Berries, consumed fresh or after processing, are a nutritionally valuable food because they are rich sources of minerals, vitamins, sugars, dietary fibres, organic acids and polyphenolic compounds. They have gained attention due to their potential to improve human health due to the presence of phytochemicals.

Their regular consumption was shown to exhibit numerous benefits such as cancer protective and anticancer effects[28,29], cardioprotective effects[30,31] or antimicrobial activities against human pathogens[32,33 34]. The different species of berries have a different efficacy in terms of their biological effects, depending on the quality and quantity of the active compounds present. Due to both the quantitative and qualitative high diversity of these bioactive compounds, berry fruits are increasingly referred to as natural functional food[35].

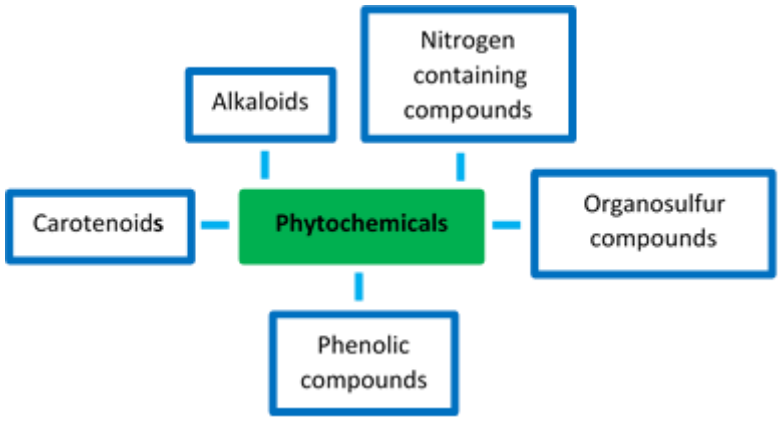

Figure 2 – The main classes of phytochemicals.

1.3.1. Phenolic compounds

Among the various phytochemicals, phenolic compounds are the largest group and the most widely distributed in the plant kingdom[36]. These secondary metabolites are also one of the most diverse in edible plants[37]. The basic structural feature of phenolic compounds is an aromatic ring bearing one or more hydroxyl groups (Figure 3)[38,39]. Plant phenolic compounds are classified as simple phenols or polyphenols based on the number of phenol units in the molecule. Thus, plant phenolics comprise simple phenols, coumarins, lignins, lignans, condensed and hydrolysable tannins, stilbenes, phenolic acids and flavonoids[38,40]. Phenolic compounds appear to largely contribute to the biological effects of plants, having many potential biology properties and extensive studies are being carried out at present on their important effects on human health [34,35]. Their biological activities are as antioxidants through free-radical scavenging activity and metal chelation, and they are also capable of modulation of enzymatic activity, inhibition of cellular proliferation and altering signal transduction pathways[1,41]. Other

experimental studies performed on animals or cultured human cell lines support that polyphenols prevent cardiovascular diseases[42,43], cancers[44,45], neurodegenerative diseases[46,47], diabetes[48,49] or osteoporosis[50]. However, it is very difficult to predict the direct effects of polyphenol intake on disease prevention in humans. One of the reasons are the doses or concentrations used in these studies, that are far beyond the ones documented in humans[41].

Interest in phenolic compounds has been focused on specific groups, one of them flavonoids. Flavonoids are divided into subgroups (flavonols, flavones, flavanones, flavanols (catechins), anthocyanins, isoflavones and chalcones)[40] and are found in many food products with plant origin such as vegetables, fruits, tea and wine[51]. They are concentrated in the seeds, fruit skin or peel, bark and flowers[52]. These compounds exhibit various physiological activities including antioxidant[53,54], anti-inflammatory[55,56], antimutagenic and anticarcinogenic[57,58].

In *C. album* berries the phenolic acids are considered to be the main group of phenolic compounds present[9]. Extracts of the berries showed to be rich in hydroxycinnamic acids (phenolic acids), and also to contain flavonoids and stilbenes[1]. However, the total phenolic content in *C. album* berries is thought to be lower than that of other coloured berries because the amount of anthocyanins is very low[9]. Anthocyanins are the flavonoids universally responsible for the colour of fruits, vegetables, cereal grains and flowers[59]. As the white crowberry is white or pinkish white it has few anthocyanins[9]. But the phenolic compounds are a very vast group of compounds and it is important to know each one that is present in these berries.

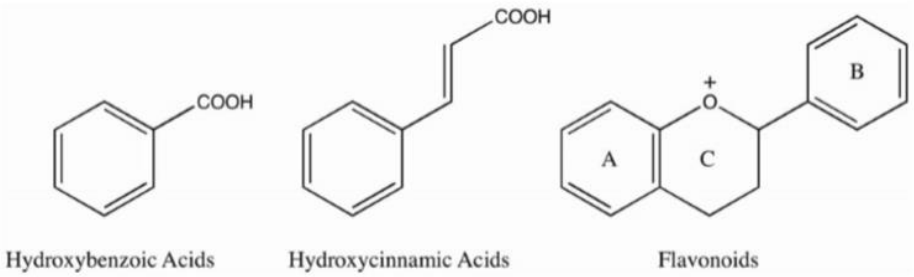

Figure 3 – Basic structures of phenolic acids (hydroxybenzoic and hydroxycinnamic acids) and flavonoids. The basic structure of flavonoids consists of two benzene moieties (rings A and B) linked in the middle through a heterocyclic pyran or pyrone with a double bond (ring C) (from [38]).

1.3.2. Alkaloids

Alkaloids are a structurally diverse group of over 12000 cyclic nitrogen-containing compounds that are found in over 20% of plant species. This chemical group has contributed to the majority of poisons, neurotoxins, and traditional psychedelics and social drugs (nicotine, caffeine, methamphetamine (ephedrine), cocaine and opiates) consumed by humans. Given their toxicity profile and low levels of efficacy in terms of benefits to brain function, only few alkaloid-based psychotropics are appropriate for use in healthy populations[60]. Some examples are the compounds galantamine and rivastigmine that are prescribed as a treatment for the Alzheimer's disease[61]. Trigonelline also has therapeutic potential because of its beneficial effects in diabetes and central nervous system diseases but further *in vivo* and *in vitro* study is required, especially with regard to its mechanisms of action[62]. In certain conditions trigonelline may also damage bone, as shown in a study performed in rats[63].

Recent research in alkaloids is considering its possible medicinal use in the treatment of depression[64] and also as an antiplatelet agent[65].

1.4. Antioxidant activity

Reactive oxygen species (ROS) are produced by living organisms as a result of their normal cellular metabolism. Environmental factors, such as air pollutants, cigarette smoke, ionizing radiation and heavy metal ions, also contribute to the production of ROS. At low to moderate concentrations, they interact in physiological cell processes, forming products like superoxide anion in mitochondria during production of adenosine triphosphate (ATP) - the molecule that provides energy for many processes in the cells[66].

ROS are produced from molecular oxygen and can be divided in two groups: free radicals and nonradicals. Free radicals are molecules containing one or more unpaired electrons that give reactivity to the molecule. When two free radicals share their unpaired electrons, nonradical forms are created. The three major ROS that are of physiological significance are superoxide anion (O_2^-), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2). They are highly reactive molecules and can damage cell structures such as carbohydrates, nucleic acids, lipids, and proteins and alter their functions. The human body as a variety of antioxidants to counterbalance the effect of oxidants since cells developed an

intricately regulated antioxidant defence system. The cellular antioxidant defence is made up of enzymatic defence, non-enzymatic defence and the DNA repair systems[66]. The mechanism of action of antioxidants consist of firstly, neutralizing their action by donating an electron to the free radical, and second the removing of ROS by quenching chain-initiating catalyst. Antioxidants may exert their effect on biological systems by different mechanisms including electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation[67]. However, when the balance between oxidants and antioxidants is in favour of oxidants the cells are under "oxidative stress". Oxidative stress contributes to many pathological conditions and diseases, including cancer[68–70], neurological disorders[71], hypertension[72], acute respiratory distress syndromes[73], chronic obstructive pulmonary disease[74] and asthma[75,76].

Phytochemicals may contribute to reduce the oxidative stress due to its antioxidant activity and capacity to trigger the cellular defence system to protect our cells against molecular damage. Dietary phytochemicals have been associated with increasing the levels of both enzymatic and non-enzymatic antioxidant defence in animal[77–79] and human dietary intervention studies[80–82]. Consumption of phytochemical-rich diets increased the expression of genes associated with DNA repair and immune responses in humans[83–85] [23]. Research suggests that the increased intake of fruits and berries may be associated with a reduced incidence of disorders induced by ROS[86,87].

A study showed that *C. album* berries extracts can help cells in a case of oxidative stress. Pre-treatment of HepG2 cells, a human liver cancer cell line, with extracts of *C. album* (1-40 μg/mL of acetone extract, ethyl acetate extract and water extract) increased their capacity to face oxidative stress (induced by t-butyl hydroperoxide, t-BOOH), greatly preventing cell damage and slightly but significantly reducing ROS, having antioxidant activity. The compounds present in the berries extracts can be responsible to enhance the quenching of the ROS generated during the period of oxidative stress[1].

1.5. Anticancer activity

Cancer can result from abnormal proliferation of any of the different kinds of cells in the body. This uncontrolled growth of cells, which can invade and spread to distant sites of the body (metastasis), is a global health problem with

high mortality and disability rates. The development of cancer involves the occurrence of mutation and the mutated cells acquire the capacity to proliferate, survive, invade, and metastasize[88]. In most cases, activation of oncogenes (specific genes capable of inducing cell transformation, as a consequence of genetic alterations that either increase gene expression or lead to uncontrolled activity of the oncogene-encoded proteins, thereby providing the first steps into cancer) and/or deactivation of tumour suppressor genes (normally acting to inhibit cell proliferation and tumour development)[88] lead to uncontrolled cell cycle progression and inactivation of apoptotic mechanisms[89]. The substances that cause cancer are called carcinogens and include radiation, chemicals and viruses. The majority (approximately 80%) of human cancers are caused by radiation and chemicals[88].

Phytochemicals can be important regulators in the processes and conditions that induce cancer. They can modulate gene expression, leading to restoration of the normal signal transduction pathways. Phytochemical intervention in chemotherapy is sustained by a higher number of clinical trials that have shown that these compounds increase the treatment efficiency and decrease the side effects, inducing apoptosis in cancer cells, reducing drug resistance and also the severity of comorbid conditions. Thus, they are a potential complementary treatment for cancer[13,27,90,91].

Many berries constituents have been suggested to exert cancer protective effects in cells, including phenolic acids (hydroxycinnamic acid, hydroxybenzoic acid), stilbenes (resveratrol, pterostilbene, piceatannol), flavonoids (anthocyanins, flavonols, catechins), lignans and tannins (proanthocyanidins, ellagitannins). Thus, berries are notably rich in compounds that are shown to exert potential for chemoprevention[28].

In this work we aim to contribute to the valorisation of the maritime plant *C. album* through the determination of the nutrient profile of its berries and addressing the phytochemical potential by quantification of total phenolic compounds content, in particular flavonoids, of extracts made with its berries and leaves. Another aim of this study was to determine the phytochemical potential of the plant by measuring the antioxidant and anticancer activity of the extracts made with its berries and leaves and to develop foods (cookies and bread) with the addition of white crowberries.

2

2. Materials and Methods

2.1. *Corema album* berries and leaves

C. album berries used throughout the study were harvested in Palheirão, Praia de Mira, a Portuguese village in the district of Coimbra, in July and frozen in plastic boxes at -75 °C (Thermo Scientific Revco Value Plus freezer). The leaves, used fresh, were harvest at the same place at the time the studies were carried out.

2.2. Preparation of *Corema album* berries and leaves

2.2.1. Preparation of berries

C. album berries were first lyophilized and reduced to powder to prepare a homogeneous sample. The process of freeze-drying is done in three main phases: first – freezing, or sub-cooling, the product; second - dehydration (drying) by ice sublimation under vacuum, and third - completion of product drying by vacuum drying[92]. Lyophilization was carried out with a condenser temperature of -98 °C at a pressure of 9.2 Pa using a freeze dryer (CoolSafe, Scanvac). A first attempt was made to lyophilize the intact berries but they were not totally dehydrated and had a sticky texture. For this reason, the berries were cut in half with a scalpel before the freeze-drying process. After freeze-dried, they were reduced to powder with a shredder. Finally, the lyophilized berries powder was stored in a refrigerator at 4 °C.

2.2.2. Preparation of leaves

The fresh leaves were reduced to small pieces using a grinder food processor (Vorwerk) and stored in a refrigerator at 4 °C.

2.3. Nutritional characterization of *Corema album* berries

The nutritional characterization was performed for the lyophilized berries powder, following the Weende or proximate analysis that was developed by Henneberg and Stohmann in 1864[93].

2.3.1. Determination of moisture and water activity of berries

The moisture content of the lyophilized berries powder was determined at 105 °C in duplicate and in accordance to NP 875:1994[94]. For this purpose, crucibles were dried at 105 °C for 2 h, cooled in a desiccator for 15 min and weighted. Approximately 2 g of the lyophilized berries sample were weighted for each crucible, and the weight value of the crucibles with the sample was recorded. The crucibles were then placed in an oven at 105 °C for 2 h. After this time the dry sample was cooled in the desiccator and subsequently weighted. The moisture is given, in percentage of dry matter (g/100 g of sample), by:

$$\text{Moisture (in dry matter)} = 100 \times \frac{P_2 - P_3}{P_2 - P_1}$$

P_1 - crucible weight (g)
P_2 - crucible weight with sample (g)
P_3 - crucible weight with dry sample (g)

For measuring the water activity (a_w) of the berries sample was used a water activity meter (Humimeter RH2, Schaller) and the value was recorded.

Moisture and water activity were also determined for the grinded leaves, following the same procedures.

2.3.2. Crude ash determination

To determine the ash content (NP 518:1986[95]), crucibles were dried at 105 °C for 2 h, cooled in a desiccator and weighted. Approximately 2 grams of the

sample were placed in the crucibles and weighted. The crucibles were placed in a muffle at 550 °C for 2 h and then cooled in the desiccator and weighted. Crude ash is given in percentage (g/100 g of sample), in dry matter and crude matter, by:

$$\text{Ash (in dry matter)} = 100 \times \frac{P_2 - P_3}{P_2 - P_1}$$

$$\text{Ash (in crude matter)} = \frac{\text{Ash (in dry matter)} \times (100 - M)}{100}$$

P_1 - crucible weight (g)
P_2 - crucible weight with sample (g)
P_3 - crucible weight with ash (g)
M - moisture (%)

2.3.3. Crude fat determination

The crude fat was determined by Soxhlet extraction with diethyl ether, in accordance with NP 876:2001[96]. For this purpose, round-bottom flasks were dried in an oven at 105 °C for 2 h, cooled in a desiccator and then weighted. The flasks were filled with diethyl ether (Sigma-Aldrich) up to 2/3 of their capacity. 2 g of sample previously dried at 105 °C were weighted and placed in an extraction thimble. The extraction thimbles were placed in the Soxhlet extractor (Behrotest R306 S, Behr Labor Technik, Fisher Scientific), the Soxhlet extraction apparatus was assembled and the extraction was carried out for 16 h. After this time, the flasks were removed from the Soxhlet extractor and the solvent was carefully collected from the apparatus. The solvent was evaporated with the aid of a rotary evaporator (Rotavapor RII, Buchi). Subsequently, the flasks were placed in the oven at 105 °C for 2 h, cooled in the desiccator and weighted. Crude fat is given in percentage (g/100 g of sample) by:

$$\text{Crude fat (in dry matter)} = 100 \times \frac{P_3 - P_1}{P_2}$$

$$\text{Crude fat (in crude matter)} = \frac{\text{Crude fat (in dry matter)} \times (100 - M)}{100}$$

P_1 - flask weight (g)
P_2 - sample weight (g)
P_3 - flask weight with fat (g)
M - moisture (%)

2.3.4. Crude fibre determination

Crude fibre was determined according to the NP 1005:1974[97]. Approximately 2 g of the sample, previously dried at 105 °C, were weighted and placed in a tall 800 mL beaker. 200 mL of sulfuric acid (12.5 g/L, Sigma-Aldrich) were added and the beaker was placed in a crude fibre apparatus (Labconco) for 40 min. With the aid of a vacuum pump, the content of the beaker was filtered into a filter plate crucible G2. The mixture was removed from the crucible, placed in the beaker and 250 mL of sodium hydroxide (12.5 g/L, Sigma-Aldrich) were added. The mixture was again placed on the crude fibre apparatus for 40 min and the filtration was also done to a filter plate crucible G2 with the aid of the vacuum pump. Subsequently, the crucible was placed in an oven at 130 °C for 2 h, cooled in a desiccator and weighted. Finally, the crucible was placed in a muffle at 550 °C for 2 h, cooled in the desiccator and weighted. Crude fibre is given in percentage (g/100 g of sample) by:

$$\text{Crude fibre (in dry matter)} = 100 \times \frac{P_2 - P_3}{P_1}$$

$$\text{Crude fibre (in crude matter)} = \frac{\text{Crude fibre (in dry matter)} \times (100 - M)}{100}$$

P_1 - sample weight (g)
P_2 - crucible weight with dry sample at 130 °C (g)
P_3 - crucible weight with dry sample at 550 °C (g)
M - moisture (%)

2.3.5. Crude protein determination

Crude protein was determined following the Kjeldahl method[98,99] (NP 2030:1996[100]). For this, 0.5 g of sample, previously dried at 105 °C, were weighted and placed in a Kjeldahl tube. 12.5 mL of sulfuric acid (Sigma-Aldrich) and a selenium catalyst (Fisher Scientific) were added. Two control assays were also

performed with the same reagents but without addition of sample. The Kjeldahl tubes were placed in a Kjeldahl digester at 400 °C for 2 h. Subsequently the tubes were left to cool in a fume cupboard and 50 mL of distilled water were added. 25 mL of boric acid solution (Sigma-Aldrich) with a mixed indicator were added to an Erlenmeyer flask and this flask, together with the Kjeldahl tube, was placed in an automatic Kjeldahl distillation unit (UDK 129 Distillation Unit, Velp Scientifica) preceding the addition of 50 mL of 40% sodium hydroxide (Sigma-Aldrich). 150 mL of distillate were collected into the Erlenmeyer flask which were then titrated with a 0.1 M hydrochloric acid solution (Sigma-Aldrich). For the crude protein calculations, the factor of 6.25 was considered. This factor is used when a specific factor is not listed and until a more appropriate factor has been determined[101]. Crude protein is given in percentage (g/100 g of sample) by:

$$\text{Crude protein (in dry matter)} = \text{factor} \times 100 \times \frac{0{,}01401 \times [\text{HCl}] \times (\text{V} - \text{V}_0)}{\text{P}_1 \times 10}$$

$$\text{Crude protein (in crude matter)} = \frac{\text{Crude protein (in dry matter)} \times (100 - \text{M})}{100}$$

P_1 - sample weight (g)
[HCl] - concentration of hydrochloric acid (M)
V - volume of titrant in the titration of sample (mL)
V_0 - volume of titrant in the titration of control (mL)
M - moisture (%)

2.3.6. Total carbohydrates determination

Total carbohydrates or non-nitrogenous extractives correspond approximately to the other elements present in the sample in addition to moisture, crude fat, crude protein, crude fibre and ash. Thus, the total carbohydrates content, in percentage (g/100 g of sample), was estimated by difference between 100 and the sum of the percentages of moisture, crude protein, crude fat and ash contents.

2.4. Preparation of *Corema album* extracts

2.4.1. Preparation of extracts of lyophilised berries

For the preparation of the extracts were weighted 5 g of the lyophilized berries powder with a ratio of plant/solvent of 1(dry basis):10. Two extracts were made: an aqueous extract with ultrapure water (BW6h) and a methanol (Sigma-Aldrich, 99.5%) extract (BM6h). The extracts were performed in a heating plate only with the stirrer on, covered with aluminium foil to protect from light exposure and left to extract for 6 h. After this time, the extracts were centrifugated in a centrifuge (High Speed Brushless Centrifuge MPW-350R) in falcon tubes at 3913 G for 10 min to remove larger particles. A vacuum filtration was then made with filter paper of 2.5 μm, followed by a new centrifugation at 35217 G for 10 min. Finally, a filtration with a syringe filter of 0.20 μm was made and the extracts were stored in a refrigerator at 4 °C covered with aluminium foil.

2.4.2. Preparation of extracts of grinded leaves

For the preparation of the extracts were weighted 16.8 g of fresh grinded leaves (the ratio used was 1:20 and not 1:10 as for the preparation of the berries extract since the grinded leaves are very light and have a high volume for the amount of water used, making its extraction difficult). Three extracts were made: two aqueous extracts with ultrapure water and a methanol extract. The extracts were performed in a heating plate only with the stirrer on and covered with aluminium foil to protect from light exposure. One of the aqueous extracts was left to extract for 16 h (LW16h) and the other for 6 h (LW6h). The methanol extract was left to extract for 6 h (LM6h). After this time, the extracts were centrifugated in falcon tubes at 3913 G for 10 min to remove larger particles. A vacuum filtration was then made with filter paper of 2.5 μm followed by a new centrifugation at 35217 G for 10 min. Finally, a filtration with a syringe filter of 0.20 μm was made and the extracts were stored in a refrigerator at 4 °C covered with aluminium foil.

2.4.3. Determination of the concentration of all extracts made

The extracts concentration (mass/volume of extract) was determined measuring the mass of a known volume of extract after drying at 65 ± 1 °C until constant weight. 1 mL of each extract was pipetted into pre-weighted Eppendorf tubes and allowed to dry in an oven at 64 °C for 3 days. Each extract was pipetted

in triplicate. After drying, they were left to cool in a desiccator for 15 min and then weighted.

2.5. Determination of total phenolic content of the extracts

To determine the total content of phenolic compounds present in the extracts of berries and leaves was followed the Singleton and Rossi (1965) method[102] with some modifications. The basic mechanism involved is an oxidation/reduction reaction. The phenolic compounds of the plant extracts suffer oxidation in alkaline solution by the yellow molybdotungstophosphoric heteropolyanion reagent Folin-Ciocalteu, transferring electrons to them and forming a blue complex (phosphotungstic/phosphomolybdenum complex) that can be quantified by spectrophotometry[103].

The experiment was performed using Folin-Ciocalteu's reagent (PanReac, AppliChem), sodium carbonate (Sigma-Aldrich) and gallic acid (Sigma-Aldrich) as the standard phenol. A 0.1 mg/mL standard solution of gallic acid in methanol:water (70:30 v/v) was made with methanol (Sigma-Aldrich, Germany) and ultrapure water. For the preparation of phenolic acid solutions for making the standard curve 10 to 100 μL (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100) of the standard solution were added to culture tubes and diluted to 100 μL with ultrapure water or methanol (ultrapure water for the standard curve of aqueous extracts and methanol for the standard curve of methanol extracts) to obtain different concentrations of gallic acid in both solvents. 100 μL of all plant extract solutions with concentrations of 250 μg/mL and 100 μg/mL were added to other culture tubes. Then 100 μL of Folin-Ciocalteu's reagent were added to each tube: the tubes with extract solutions with two different concentrations, the standard solutions and also the blank (consisting of 100 μL of ultrapure water for the aqueous extracts and 100 μL of methanol for the methanol extracts). The mixture was mixed in a vortex and placed in a thermostatic water bath (at 40 °C) for 4 min. After this time, 800 μL of a 5% (w/v) sodium carbonate solution were added to the tubes and the mixture was mixed in the vortex and returned to the bath. The reaction was kept in the dark. After 20 min the tubes were rapidly cooled in cold water and the total phenolic content was determined colorimetrically by measuring the absorbance at 750 nm in a microplate reader (BioTek μQuant MQX200)

using a 48 well culture plate. These determinations were performed in duplicate. The total phenolic content of the extracts was determined from the calibration curve and expressed as gallic acid equivalents (mg) per gram of extract.

2.6. Determination of flavonoid compounds of the extracts

The content in flavonoids was determined by the Al-Dabbas *et al.* (2006) method[104] with slight modifications. This method uses aluminium chloride ($AlCl_3$) as the reagent and is based on the reaction of aluminium ions with flavonoid molecules under basic conditions. The products of this reaction are red aluminium flavonoid chelates capable of absorption under a certain wavelength. Colour intensity and method sensitivity depends on the number of hydroxyl groups in the flavonoid molecule and on the properties of the aluminium ion[105].

A methanolic solution of quercetin (methanol and quercetin from Sigma-Aldrich, Germany) with a concentration of 0.1 mg/mL was used as the standard for a calibration curve. For making the standard curve 10 to 100 μL (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100) of the methanolic solution of quercetin were added to a 96 well culture plate and diluted to 100 μL with methanol to obtain different concentrations of quercetin in this solvent. 100 μL of all plant extract solutions with concentration of 1 mg/mL were mixed with 100 μL of 2% $AlCl_3$ (Sigma-Aldrich) in methanol. The same volume of 2% $AlCl_3$ solution was also added to all the wells containing the standard solutions of quercetin. The mixture was then left for 40 min at room temperature and its absorbance was measured at 415 nm in a microplate reader. In the blank was used a mixture of 100 μL of methanol with 100 μL of 2% $AlCl_3$ solution, followed by measurement of the absorbance as described above. The analysis was performed in triplicate. The total amount of flavonoids was determined from the calibration curve and expressed as quercetin equivalents (mg) per gram of extract.

2.7. Determination of antioxidant activity of the extracts

The potential antioxidant activity of the extracts of berries and leaves was determined by the DPPH free radical scavenging method. This method was described by Brand-Williams *et al.* (1995) and involves the use of the free radical

2,2-diphenyl-1-picrylhydrazyl (DPPH•)[106,107]. The DPPH solution has a dark violet colour that when mixed with antioxidants starts to decrease in intensity because the antioxidants react with the stable radical in a methanolic solution. This change is a sign of resistance to the oxidative stress induced in the medium. This reaction of antioxidants with the DPPH radical can occur by transfer of electrons or hydrogen atoms from the antioxidants to the radical, forming a stable molecule. The reduction in the concentration of the DPPH• is monitored by the decrease in the absorbance at a characteristic wavelength, 515 nm, due to the reduction of colour intensity. This wavelength is used because it corresponds to the maximum absorbance of DPPH in its radical form[106,108].

The method was performed using DPPH (Sigma-Aldrich) and methanol (Sigma-Aldrich, Germany). A DPPH solution of 250 μM in methanol was made in the day of the experiment. To ensure the complete dissolution of the DPPH in methanol, the solution was stirred with a magnetic stirrer for 20 min while kept in the dark with aluminium foil. After made, the DPPH solution was stored in the refrigerator. For this experiment six concentrations of all the 5 extracts made were used: 250 μg/mL; 100 μg/mL; 50 μg/mL; 25 μg/mL; 10 μg/mL and 5 μg/mL. The concentrations were chosen according to their antiradical capacity towards DPPH radicals based on two pilot determinations made. For the preparation of the controls 100 μL of the solvent used to make the extracts (ultrapure water for the aqueous extracts and methanol for the methanol extracts) were added to culture tubes. For the preparation of the culture tubes with the extracts, 100 μL of each one of the 5 extracts were pipetted into them, in the six concentrations mentioned. Subsequently, 100 μL of the DPPH solution were added to each tube, the mixture was mixed in a vortex and kept away from the light at room temperature. After 30 min of reaction, the absorbance was measured at 515 nm in a microplate reader using a 96 well culture plate. These determinations were performed in triplicate. For each extract the blank was also prepared: 100 μL of the extract and 100 μL of the solvent used to prepare the DPPH solution, methanol. The mixture in the culture tubes was mixed in the vortex and the absorbance at 515 nm measured in a microplate reader using a 96 well culture plate. These determinations were also performed in triplicate. The percentage of antioxidant activity (AA) of each extract concentration was calculated using the following equation:

$$\% AA = \frac{A_{control} - (A_{extract} - A_{blank})}{A_{control}} \times 100$$

Where $A_{control}$ is the absorbance of control, $A_{extract}$ is the absorbance of extract and A_{blank} is the absorbance of blank. The antioxidant activity of each extract was defined as the concentration of extract necessary to decrease the initial concentration of DPPH free radicals by 50%: the half minimal inhibitory concentration or IC_{50}. This concentration value was obtained by interpolation and using linear regression analysis.

2.8. High-performance liquid chromatography analysis of phenolic compounds

Chromatography is an important biophysical technique that enables the separation, identification and purification of the components of a mixture for qualitative and quantitative analysis. The technique is based on the principle where molecules in mixture applied onto a surface or into a solid, called stationary phase, can be separated from each other while moving with the aid of a mobile phase. Thus, three components form the basis of the chromatography technique:

- Stationary phase: composed of a "solid" phase or "a layer of a liquid adsorbed on the surface of a solid support";
- Mobile phase: composed of "liquid" or a "gaseous component";
- Separated molecules.

The type of interaction between stationary phase, mobile phase and substances contained in the mixture is the basic component effective on separation of molecules from each other. The factors involved in the separation process include molecular characteristics related to adsorption, partition and affinity, or differences among their molecular weights. Because of these differences some components of the mixture stay longer in the stationary phase as they move slowly in the chromatography system, while others pass rapidly into the mobile phase and leave the system faster[109,110].

High-performance liquid chromatography (HPLC) is an instrumental form of liquid chromatography that uses stationary phases consisting of small

particles, thereby achieving more efficient separations than those used in conventional liquid chromatography. To perform this chromatography some instrumental equipment is needed. The basic equipment consists of a column packed with a stationary phase, a driving force to propel the solvent through the column (pump), a system (injector) for introducing the sample into the column, a system (detector) for measuring a physical property of the solutes being analysed that differs from the properties of the solvent or a property of the mobile phase which is altered by the presence of the solute, and a system for recording the detector signals and converting them into graphic traces of chromatograms[111].

In this work a reverse phase chromatography was performed. This chromatography uses resins with small hydrophobic groups attached. The stationary phase is of a nonpolar nature and the mobile phase is more polar than the stationary phase. The most widely used stationary phase is silica-based and the mobile phase is usually water with an organic modifier (as acetonitrile, for example). The organic modifiers are added to the elution buffer to decrease the water concentration in the mobile phase. This in turn weakens the hydrophobic attraction of the hydrophobic groups on the chromatography matrix for the compounds. The stronger hydrophobic interactions will require higher concentrations of organic modifiers. Therefore, a gradient of organic solvent will release the compounds from the column matrix in the order of their hydrophobic interaction strengths[111,112].

To perform the HPLC was necessary to previously extract the compounds present in the lyophilized berries powder and grinded leaves. Extraction of the compounds was done according to Nour *et al.* (2013) with some modifications[113]. About 0.5 g of each sample was extracted with 25 mL of methanol (Sigma-Aldrich, Germany), vortexed for 1 h and then centrifuged at 9000 G for 20 min at 4 °C, followed by evaporation in vacuum at 30 °C. The final residue was resuspended in 2 mL of methanol, filtered through a 0.45 μm filter and 20 μL were injected into the HPLC. The chromatography was performed on a Beckman System Gold (USA) HPLC equipment, equipped with solvent system (pumps) model 126, photodiode detector model 168 and data processing program 32 Karat version 8.0. A column Spherisorb ODS 2.5 μm, 250 x 4.6 mm (Waters, USA), a silica-based reversed-phase C18 column, was used at 272 nm and 320 nm. Elution was performed using a mobile phase mixture of 5% (v/v) acetic acid solution in water (solvent A) and acetonitrile (solvent B). The solvent gradient changed from 100% to 95% A in 5 min, 95% to 87% A in 5 min, maintained at 87% A for 35 min

and changed from 87% to 95% A in 1 min. The column was stabilized for 5 min before re-injection[114]. Chromatographs were acquired at λ_{max} of 272 nm and 320 nm, depending on the compounds. The alkaloid trigonelline and the phenolic acids (hydroxycinnamic acids) caffeic acid, p-coumaric acid and ferulic acid were identified and quantified according to the standard curves of each standard, obtained using different concentrations of each compound, and were already available in the laboratory (in appendix, Figure 1A). The identification and quantification of the polyphenol chlorogenic acid (3-O-caffeoylquinic acid or 3-CQA) and its isomers, cryptochlorogenic acid (4-O-caffeoylquinic acid or 4-CQA) and neochlorogenic acid (5-O-caffeoylquinic acid or 5-CQA), was made according to Trugo and Macrae (1984)[115]. Chlorogenic acids are obtained through an esterification reaction between quinic acid and caffeic acid (Figure 4)[116]. Esterification can occur on carbon 1, 3, 4 or 5, resulting in the synthesis of four different isomers, among them the isomers mentioned above[117]. For the isomerisation of the chlorogenic acids 5-CQA (Sigma-Aldrich), 200 mg were dissolved in distilled water (20 mL) and the pH was adjusted to 8 with dilute ammonia (Sigma-Aldrich) solution. This solution was heated for 30 min in a boiling water-bath. The pH was then adjusted to 2.5-3.0 with dilute hydrochloric acid (Sigma-Aldrich), after cooling to room temperature. Quantification was achieved by peak-area measurement and by comparison with a 5-CQA standard. It was possible to quantify the individual isomers using their molar absorptivities, according to the equation:

$$C = \frac{RF \times \varepsilon_1 \times M_{r_2} \times A}{\varepsilon_2 \times M_{r_1}}$$

where C is the concentration of the isomer in mg/mL; RF is the response factor of 5-CQA standard (concentration mg/mL/mAU); ε_1 is the molar absorptivity of 5-CQA; ε_2 is the molar absorptivity of the isomer in question (3 or 4-CQA); M_{r1} is the relative molecular mass of 5-CQA; M_{r2} is the relative molecular mass of the isomer in question; and A is the area of the peak corresponding to the isomer in question[115]. Molar absorptivities of 3-CQA, 4-CQA and 5-CQA at 320 nm were determined in three replicate measurements. Calibration graphs were plotted using the CQA isomer mixtures diluted at five different concentrations. Recoveries were checked by the method of standard additions. The levels of addition to the samples (in grams of isomer per 100 g of sample) were 3-CQA: 0.3-1.6; 4-CQA: 0.3-1.5; 5-CQA: 0.3-1.7.

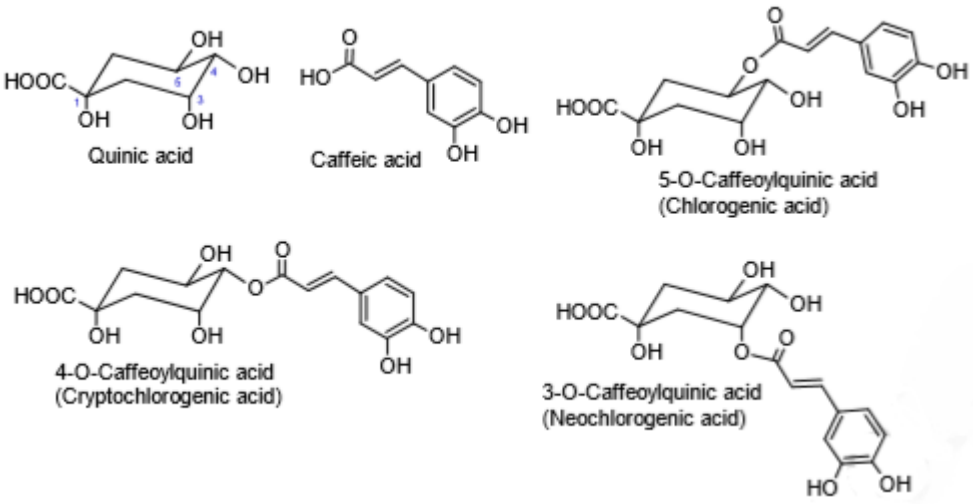

Figure 4 – Structures and nomenclature of the common mono-caffeoylquinic acids and their building blocks, quinic acid and caffeic acid (trivial names in brackets) (from [126]).

2.9. Infrared spectroscopy: attenuated total reflection measurements

Infrared spectroscopy (IR) is used for studying biological molecules and can be applied to study any sample in any state: liquids, solutions, pastes, powders, films, fibres, gases and surfaces. This technique is based on the vibrations of the atoms of a molecule. Thus, an infrared spectrum is obtained by passing infrared radiation through a sample and determining what fraction of the incident radiation is absorbed at a particular energy. The energy at which any peak in an absorption spectrum appears corresponds to the frequency of a vibration of a part of the sample molecule.

The attenuated total reflection (ATR) Fourier transform infrared (FTIR) spectroscopy (ATR-FTIR) involves directing the infrared light at an interface between an infrared transparent material with a high refractive index called the internal reflection element (IRE, a prism made of diamond or germanium, for example) and a sample on the surface of the IRE[118]. The IRE is designed so that the infrared beam of light strikes on the plate at an angle greater than the critical angle, resulting in total internal reflection. Under these conditions, the beam intensity is attenuated by a surface 'evanescent' wave that penetrates a short distance into any absorbing sample placed in contact with the crystalline plate. The depth of penetration (d_p) varies with the angle of incidence (θ), the wavelength (λ) and the indices of refraction of both the plate (n_1) and the sample (n_2), according to the equation[119,120]:

$$d_p = \frac{\lambda}{2\pi(n_1^2 \sin^2\theta - n_2^2)^{\frac{1}{2}}}$$

The resulting spectrum is similar to a transmission spectrum, with some differences in the intensity of the peaks because of the variable penetration[119].

To study the composition of the lyophilized berries powder and grinded leaves by infrared spectroscopy an attenuated total reflectance (ATR) infrared spectrometer with Fourier transform (FTIR) was used (Vertex 70 spectrometer with a Platinum ATR, both from Bruker, Billerica, Massachusetts, EUA), that was continuously purged with nitrogen gas to reduce the content of unwanted atmospheric interferents (water vapor and carbon dioxide, for example) inside the spectrometer significantly, avoiding high noise in the spectrum. The spectres were collected in the wavenumber range from 4000 cm^{-1} to 400 cm^{-1}. The samples in study were placed in direct contact with the diamond crystal and the average of 100 scans were recorded for each sample.

2.10. Cell studies

The anticancer effect of extracts of white crowberries and leaves was evaluated against the human colon adenocarcinoma HT-29 cell line. The cell line was provided by the professor Ana Margarida Urbano (PhD, professor at Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia of Universidade de Coimbra). This is an adherent cell line which grows in monolayer[121]. For maintenance, the cells were stored in an incubator (Sanyo) at 37 °C with 72-90% humidity, 5% CO_2 and 95% air.

This study was performed for the BW6h and LW6h extracts, so both extracts were obtained with the same time of extraction and because the water does not offer any type of toxicity to the cells. The BW6h extract was used after dilution in PBS to 10 mg/mL and the LW6h extract was used in its initial concentration, 15.1 mg/mL. The concentrations used were chosen taking into account that the volume of extract applied to the plates corresponds to 10% (v/v) of the total volume of the well and four successive dilutions of each extract are made. So, the concentrations of BW6h extract tested were 1.00, 0.50, 0.25, 0.13 and 0.06 mg/mL and the concentrations of LW6h extract tested were 1.50, 0.75, 0.38, 0.19 and 0.09 mg/mL.

2.10.1. Preparation of the culture medium and solutions

The HT-29 cells were grown in McCoy's 5A medium (Sigma-Aldrich) with 10% fetal bovine serum (FBS, Gibco) and 2.2 g of sodium bicarbonate ($NaHCO_3$, Sigma-Aldrich) per litre at pH 7.4. After preparation, the culture medium was filtered under vacuum with a 0.2 μm membrane filter under sterile conditions in a laminar flow biological safety cabinet (Biowizard, Kojair) and stored at 4 °C. All necessary solutions were also prepared: phosphate buffered saline (PBS), made with 1.5 mM of monopotassium phosphate (KH_2PO_4), 8.1 mM of disodium phosphate (Na_2HPO_4), 0.14 mM of sodium chloride (NaCl) and 2.7 mM of potassium chloride (KCl) (all from Sigma-Aldrich) per 500 mL in ultrapure water at pH 7.4; MTT (Sigma-Aldrich) with the concentration of 5 mg/mL in PBS 1x; SRB 0.5% (v/v) made with SRB (Sigma-Aldrich) dissolved in 1% (v/v) of acetic acid (Sigma-Aldrich); and 190 mM tris buffer made with tris(hydroxymethyl)aminomethane (Sigma-Aldrich) dissolved in ultrapure water at pH 10.

2.10.2. Subculturing and measuring cell growth

The cell line used was normally divided twice a week with a split ration of 1:8. For subculturing, the culture medium was removed from the flask and the cells washed with 5 mL of 1x PBS. After this washing step the PBS was removed, 2 mL of trypsin 1x (Sigma-Aldrich) were added and the enzyme was left to incubate for 5 min at 37 °C in the incubator. Then the mixture was resuspended with 5 mL of culture medium. According to the split ratio used, the required volume of cells and medium were added to the flask, which was then incubated.

For measuring the cell growth, the cells were counted at the beginning of each experiment with a Neubauer hemacytometer and using the trypan blue dye (0.4% (v/v), Sigma-Aldrich). For this the same steps as for subculture were followed, but after the cells were trypsinized and resuspended with 5 mL of culture medium they were washed by centrifugation at 189.4 G for 5 min. The supernatant was removed, and the cells resuspended in 10 mL of medium. In an Eppendorf 500 μL of cell suspension were diluted in 500 μL of cell medium and a 1:1 dilution of this diluted cell suspension in trypan blue was made. The cells were then resuspended, and the volume placed in one of the hemacytometer chambers. This procedure was repeated to the other chamber of the hemacytometer. The viable cells were counted under a microscope (10x, Olympus CKX41) by counting all the cells that excluded the dye. The number of cells/mL is obtained by multiplying the average of the number of cells counted in the squares (eight

in total) of the hemacytometer chambers by 10^4, obtaining the number of cells per 0.5 mL, thus being possible to determine the number of cells in 1 mL.

2.10.3. Filtration of extracts of berries and leaves

To be used in the study in HT-29 cells the BW6h and LW6h extracts had to be filtered under sterile conditions with a 0.2 μm syringe filter and diluted in PBS 1x to the final desired concentration for the experiment (10 mg/mL for the BW6h extract and 15.1 mg/mL for the LW6h extract).

2.10.4. Determination of the concentration of extracts after filtration

To determine the exact concentration of the BW6h and LW6h extracts after being filtered with 0.2 μm filters under sterile conditions the procedure to determine their concentration was repeated. 1 mL of each extract was pipetted into pre-weighted Eppendorf tubes and allowed to dry in an oven at 62°C for 3 days. Each extract was pipetted in triplicate. After drying, they were left to cool in a desiccator for 15 min and then weighted.

2.10.5. Evaluation of cellular viability and proliferation

Cell proliferation was evaluated using sulforhodamine B (SRB) colorimetric assay and cell viability using the method of reducing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or thiazolyl blue tetrazolium bromide (MTT) to determine the effect of BW6h and LW6h extracts against the HT-29 cell line and if it is dosage and time dependent. To determine the possible relationship between the effect on the cancer cells and the dosage a range of concentrations of both extracts were tested. The time dependence was evaluated by MTT and SRB assays at three different times: 24, 48 and 72 h. To validate the results three independent assays were made, and each one performed in triplicate.

- MTT assay

The MTT colorimetric assay is based on the reduction of MTT into formazan crystals by living cells. This process is carried out by the mitochondria present in the cells, which means that only viable cells with active metabolism are able to convert MTT to the purple coloured compound formazan. When cells die, they lose the ability to convert MTT into formazan, so colour formation serves as a marker of only the viable cells and can be used to measure the possible *in vitro*

cytotoxic effect of the water extracts of berries and leaves on HT-29 cells. The purple coloured formazan crystals have an absorbance maximum near 570 nm making possible the measurement by spectrophotometry[122–124].

For the MTT assay the cell line used was plated at a density of 3×10^4 cells/cm^2 in 24 well plates (with an area of 1.95 cm^2), adding 1 mL of cell suspension after adequate dilution so each well had the desired cell density. Plates with the cells were incubated at 37 °C with 72-90% humidity and 5% CO_2. After 24 h, the culture media was substituted by 750 μL of new one, except for the wells in which the extract was applied at the highest concentration tested, to which 1350 μL of medium and 150 μL of the extract tested, BW6h or LW6h, were added. 750 μL of this well were pipetted to the next well, resuspended, and the same procedure was repeated for the following three wells - successive dilutions, being possible to test five different extract concentrations (1.00, 0.50, 0.25, 0.13 and 0.06 mg/mL for the BW6h extract and 1.50, 0.75, 0.38, 0.19 and 0.09 mg/mL for the LW6h extract). Controls were also made for each time, 24, 48 and 72 h, with 1 mL of culture medium and without addition of extract. The plates were then incubated again under the same conditions and the cells were exposed to the extracts for 24, 48 and 72 h. After the specific times of exposure to the extract, the wells content and the respective control were aspired, the cells were washed with 500 μL of PBS and 500 μL of MTT (5 mg/mL in PBS 1x) were added to each well and allowed to incubate for 3 h, with the plates covered with aluminium foil. Supernatants were removed, 500 μL of dimethyl sulfoxide (DMSO, Sigma-Aldrich) were added, the plates were gently shaken to solubilize the formazan crystals and absorbance was measured at 570 nm using BioTek MQX200 μQuant microplate reader. The values of cell viability (in %) were determined according to the following equation[124]:

$$\text{cell viability (\%)} = \frac{A_{sample}}{A_{control} - A_{blank}} \times 100$$

A_{sample} - sample absorbance
$A_{control}$ - control absorbance
A_{blank} - blank absorbance

The results of percentage of cell viability were expressed in relation to the control, considered to be always 100%, and plotted in a graphic with cell viability

as a function of the concentration of BW6h and LW6h extracts applied after 24, 48 and 72 h of incubation. The extract concentration values for which 50% inhibition of cancer cells (IC_{50}) was verified, against control cultures, were determined using the program GraphPad Prism 6.01 for Windows (GraphPad Software, USA). The presented results are the mean ± standard deviation (SD, corresponding error bars are plotted in the graphs) of at least three independent assays, each one performed in triplicate. Statistical analysis was performed using the One-Way ANOVA, followed by Turkey's multiple comparisons test. Differences were considered statistically significant with: $p < 0.05$; $p < 0.01$; $p < 0.001$ or $p < 0.0001$.

- SRB assay

The SRB colorimetric assay, developed by the National Cancer Institute of the United States of America[125], was used in this study for cell density determination and is based on the measurement of cellular protein content. The SRB compound is capable of binding to protein components of the cells that have been fixed to culture plates with acetic acid. SRB is an aminoxanthene dye with a bright pink colour and has two sulfonic groups that bind to basic amino acid residues when under mild acidic conditions. The chemical bond formed can be broken under basic conditions and, as the binding of SRB to the amino acids is stoichiometric, the amount of dye extracted from the stained cells is directly proportional to the cell mass (and then linear with cell density), and this method can be used to determine cell density[124,126].

For the SRB assay were followed the same procedures of cell plating and addition of BW6h and LW6h extracts as for the MTT assay. Controls were also made for each time, 24, 48 and 72 h, and for time 0 h, with 1 mL of culture medium and without addition of extract. The plates were then incubated again under the same conditions and the cells were exposed to the extracts for 24, 48 and 72 h. After the specific times of exposure to the extract (except the 0 h time control that was aspired and washed on the day the extracts were added, after 24 h of incubation of the cells), the wells content and the respective control were aspired, the cells were washed with 500 μL of PBS followed by washing with ultrapure water (adding a few drops to each well and aspirating soon) and the plates were placed back into the incubator to evaporate the water. After all plates dried, the cells were fixed with 1% acetic acid (Pronalab) in methanol (Sigma-Aldrich,

Germany) (v/v) solution and placed at -20 °C for approximately 24 h. The 1% acetic acid in methanol (v/v) solution was removed and the plates were left to dry at room temperature. The plates were then incubated for 1 h with 250 μL of 0.5% SRB (sulforhodamine B sodium salt, Sigma-Aldrich) in 1% acetic acid (v/v), thoroughly washed with 1% acetic acid solution until removing all the unbounded dye and allowed to dry at room temperature. 500 μL of 10 mM Tris buffer solution were added to each well and absorbance was measured at 540 nm using a microplate reader. The values of cell proliferation (in %), measure by the % of SRB retention, were determined according to the following equation[124]:

$$\text{cell proliferation (\%)} = \frac{A_{\text{sample}} - A_{0\,\text{h}}}{A_{\text{control}} - A_{0\,\text{h}}} \times 100$$

A_{sample} - sample absorbance
$A_{0\,h}$ - 0 h absorbance
$A_{control}$ - control absorbance

The results were expressed as a percentage of SRB retention in relation to control cell cultures at time 0 h (C_0) and plotted in a graphic with SRB retention as a function of the concentration of BW6h and LW6h extracts applied after 24, 48 and 72 h of incubation. The extract concentration values for which 50% inhibition of cell growth (IC_{50}) was verified, against control cultures, were determined using the program GraphPad Prism 6.01 for Windows. The presented results are the mean ± standard deviation (SD, corresponding error bars are plotted in the graphs) of at least three independent assays, each one performed in triplicate. Statistical analysis was performed using the One-Way ANOVA, followed by Turkey's multiple comparisons test. Differences were considered statistically significant with: $p < 0.05$; $p < 0.01$; $p < 0.001$ or $p < 0.0001$.

2.11. Sensory evaluation of cookies and bread with white crowberries

For valuing the white crowberries and give people a better knowledge of their sensory properties and existence in our country, cookies and bread were developed with the addition of them and these products were evaluated by people in a sensory evaluation.

2.11.1. Experimental samples

To make the cookies was used the lyophilized berries powder and for the bread were used the intact berries stored frozen at -75 °C after thawing.

- White crowberry cookies

To make the cookies was followed a recipe for butter cookies with the addition of varying amounts of white crowberries powder: 1,2 or 3 g. The base recipe for the cookies was as follows: 40 g of melted butter were mixed with 60 g of sugar and the amount of white crowberries powder mentioned above. Then 160 g of flour and one egg were added to the mixture, manually mixed in the form of a ball and the cookie dough was left in the fridge for 30 min. After this time the cookie dough was rolled out, cut into circular shapes with a cookie cutter and the cookies were baked for 8 min at 200 °C. For the sensory evaluation were also made butter cookies following the same recipe but without the berries powder (standard cookies).

- White crowberry bread

To make the bread was followed a traditional wheat homemade bread recipe with the addition of varying amounts of white crowberries: intact berries (30% w/w) or grinded berries (20, 30 or 40% w/w). The base recipe for the bread was as follows: 1 kg of wheat flour was mixed with 10 g of compressed baker's yeast dissolved in 600 mL of warm water. The bread dough was kneaded with the hands and left to leaven for 1 h. After this time, the bread dough was divided into 100 g pieces and the berries were added and mixed in the dough. The bread was then baked in a wood oven for 1 h. In the same way as for the cookies, for the sensory evaluation of the white crowberry bread was also made a bread following the same recipe but without the berries (standard bread).

2.11.2. Sensory evaluation: consumer acceptability and intention to buy

For the consumer acceptability of the sensory evaluation was developed a quantitative analysis questionnaire in which the panelists gave a value (on a scale of 1 to 9: 1 - really dislike; 2 - dislike very much; 3 - moderately dislike; 4 - slightly dislike; 5 - did not like or dislike it; 6 - slightly like; 7 - moderately like; 8 - like very much; 9 - really like) to rate the sensory attributes of the cookies and bread (visual aspect, colour, smell, taste, texture and global appreciation) with and

without the addition of berries. The panelists could also write comments about the product. To determine the consumer intention to buy the same questionnaire contained a question about the intention to buy, in which one the panelists also gave a value based on their opinion about the products (on a scale of 1 to 5: 1 - certainly would not buy; 2 - probably would not buy; 3 - maybe would buy, maybe would not buy; 4 - probably would buy; 5 - certainly would buy). The panel was composed of students and employees of the Coimbra College of Agriculture (40 panelists in the cookies sessions and 38 panelists in the bread sessions). The sessions were carried out at the same location in a room specifically designed for sensory analysis, in individual booths under artificial white light at a temperature around 22 °C. Before the start of the session was given the necessary information about the foods to be tested and also about the berries, since the panelists were not familiar with products containing white crowberries and most of them reported to not know the berries in study. In each sensory analysis session was given to the panelists a sample of the cookies with and without the berries and a sample of the bread with and without the berries. All the samples were coded with 3 numbers, presented on plastic plates and the evaluation of the cookies and the bread was done separately. Cookies and bread samples were all prepared in the day before the sensory analysis. The two different samples of cookies were kept separated in boxes to avoid mixing of flavours. The two different bread samples were also kept separated in cloth bread bags for better maintenance of their characteristics. The panelists rinsed their mouths with mineral water before testing each sample.

3

3. Results and Discussion

3.1. Preparation of *Corema album* berries and leaves

As referred in materials and methods section, the process of lyophilization was first carried out with intact berries but they were not totally dehydrated. The explanation we found for this lyophilization result is that the skin of the berries is very thick, preventing a greater exit of water contained within the berry. For this reason, they were cut in half and introduced again in the freeze dryer to continue the drying process. The amount of water present was drastically reduced, as shown by the moisture and water activity (a_w) values present on Table 1. This table also shows the leaves moisture and water activity.

Table 1 – *C. album* berries and leaves moisture and water activity.

Part of the plant	**Moisture (g/100 g of sample)**	**a_w**
Berries (lyophilized)	54.51 (with intact skin) 21.11 (after being cut)	0.69 (with intact skin) 0.21 (after being cut)
Leaves (fresh grinded)	69.90	0.72

The values in the table for the berries show that there was a significant decrease in the moisture and water activity of the lyophilized berries with cut skin (21.11% and 0.21, respectively) when compared to the lyophilized berries with the intact skin (moisture of 54.51% and water activity of 0.69), which

indicates that the skin actually hinders the exit of water from the berries during the lyophilization process.

3.2. Nutritional characterization of berries

The nutritional characterization of the lyophilized berries powder following the Weende or proximate analysis is shown in Table 2. The nutritional characterization of another batch of berries preserved by freezing at -75 °C was already available (Luís Simões), following the same method (Table 3).

Table 2 – Nutritional characterization of lyophilized *C. album* berries powder obtained using the Weende analysis.

	Chemical composition (g/100 g of sample)	
	In dry matter	**In crude matter**
Moisture	–	21.11
Crude ash	1.65	1.30
Crude fat	4.00	3.16
Crude fibre	30.14	23.78
Crude protein	5.02	3.96
Total carbohydrates	59.19	46.69

Table 3 – Nutritional characterization of *C. album* berries preserved by freezing at -75 °C obtained using the Weende analysis [Luís Simões, unpublished].

	Chemical composition (g/100 g of sample)	
	In dry matter	**In crude matter**
Moisture	–	84,79
Crude ash	2,01	0,31
Crude fat	4,77	0,72
Crude fibre	42,68	6,49
Crude protein	5,99	0,91
Total carbohydrates	44,56	6,78

The main components in the lyophilized berries powder are total carbohydrates, followed by crude fibre. The lyophilized berries are low in crude ash and also have a reduced fat content, by comparison with the other components present. The protein content is slightly higher than the fat content. Therefore, lyophilized white crowberries are a good food source of total carbohydrates and fibres.

By comparing the nutritional characterization made to the lyophilized berries with the nutritional characterization already done to the berries preserved by freezing at -75 °C, in terms of dry matter, the values of the chemical composition should be similar since water is the only component that is removed during the lyophilization process. The chemical composition showed to be close for almost all compounds, being the lyophilized berries richest in total carbohydrates (59.19% in the lyophilized berries and 44.56% in the preserved berries) and less rich in crude ash (1.65% in lyophilized and 2.01% in preserved), crude fat (4.00% in lyophilized and 4.77% in preserved), crude fibre (30.14% in lyophilized and 42.68% in preserved) and crude protein (5.02% in lyophilized and 5.99% in preserved).

3.3. Determination of the concentration of lyophilized berries and grinded leaves extracts

The concentration of the two lyophilized berries powder extracts (BW6h and BM6h) and the three grinded leaves extracts of *C. album* (LW16h, LW6h and LM6h) is shown in Table 4.

Table 4 – Concentration of *C. album* lyophilized berries powder and grinded leaves extracts made with the solvents water and methanol.

Extracts	**Mean concentration (mg/mL)**
BW6h	49.6
BM6h	54.6
LW16h	13.9
LW6h	15.1
LM6h	40.0

For both grinded leaves and lyophilized berries powder, the extracts made with methanol as solvent had a higher concentration than the extracts made with water. In terms of the time of extraction, the leaves extract in water with 16 h of extraction did not result in a higher final concentration (13.9 mg/mL) than the extract made with 6 h of extraction (15.1 mg/mL).

3.4. Determination of total phenolic content of the extracts

The total phenolic content of lyophilized berries extracts and grinded leaves extracts in water and methanol was determined using the Singleton and Rossi (1965) method[102] with some modifications and the calibration curves of aqueous and methanolic gallic acid solutions are present in Figures 5 and 6, respectively.

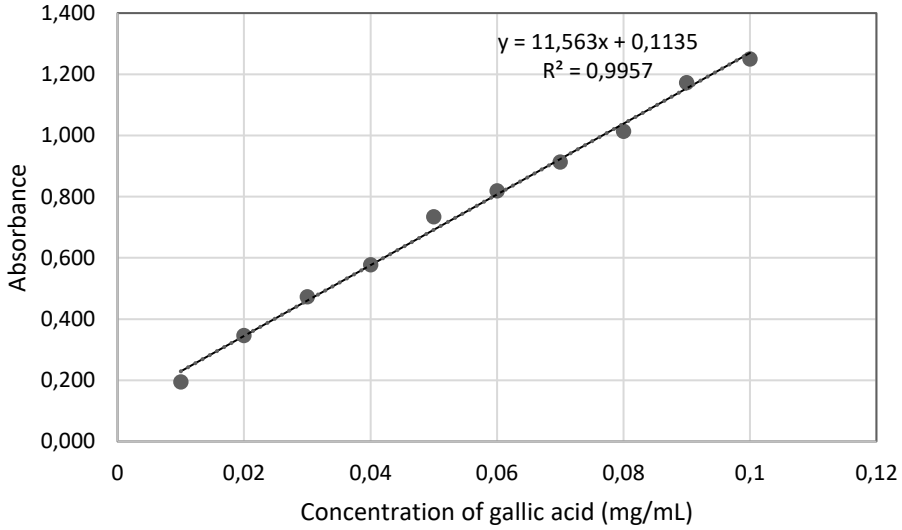

Figure 5 – Standard curve of aqueous gallic acid solution.

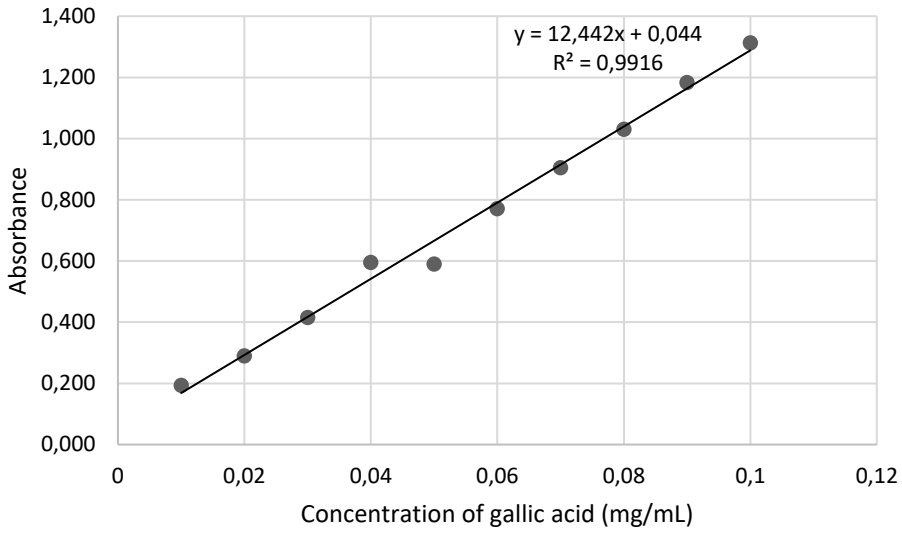

Figure 6 – Standard curve of methanolic gallic acid solution.

Comparing both calibration curves is possible to conclude that the solvent used (water or methanol) has no great influence on the gallic acid standard curve obtained.

Using the equations of the standard curves was possible to determine the equivalent amount of gallic acid present in each extract (mg/mL). By multiplying this value by the inverse of the extract concentration was obtained the concentration of gallic acid in mg per mg extract. The total phenolic content in the extracts is expressed as mg of gallic acid equivalents per g of extract and the results are shown on Table 5.

Table 5 – Total phenolic content in the *C. album* extracts expressed in milligrams of gallic acid equivalents per gram of extract (mg/g).

Extracts	**Gallic acid equivalents per gram of extract (mg/g)**
BW6h	3.1
BM6h	29.7
LW16h	176.2
LW6h	258.5
LM6h	333.3

For both solvents used in the extraction, water and methanol, and for the same time of extraction (6 h), the amount of phenolic compounds in mg present

per g of extract is much higher in the leaves (258.5 mg/g in the water extract and 333.3 mg/g in the methanol extract) than in the berries (3.1 mg/g in the water extract and 29.7 mg/g in the methanol extract). This result suggests that *C. album* leaves extract has a higher potential as a source of phenolic compounds than the berries extract.

Also, the amount of phenolic compounds present in the other water leaves extract showed that the attempt to make a leaf extract in water for a longer time (16 h) did not result in an increase of the amount of phenolic compounds extracted. In fact, the opposite happened, the amount of phenolic compounds extracted reduced, as it is possible to see when comparing the extract of leaves in water with a time of extraction of 16 h (176.2 mg/g) with the other leaves extract also in water but with a time of extraction of 6 h (258.5 mg/g). This reduction may also suggest that the extraction of the leaves with water over a long period of time can be accompanied by the beginning of degradation of compounds present in the leaves.

According to Leon-González *et al.* (2013), the total phenolic content of the fresh white crowberries, using the same method but performing the extraction in acetone, was 1.2 mg/g[9]. This lower value of phenolic compounds may be due to the use of fresh berries, whereby a high amount of water is present, and the phenolic compounds are diluted in that water, and may also be due to the use of a different solvent for the extraction, acetone.

The effect of the solvent used for the preparation of the extracts in the extraction of phenolic compounds from the berries and leaves of *C. album*, comparing only the extracts obtained with the same extraction time (6 h), is illustrated in Figure 7. The results suggest that the solvent used has an effect on the extraction of the phenolic compounds present, from both berries and leaves. For the leaves, the extraction of the phenolic compounds with methanol was superior to that achieved with water (333.3 mg/g and 258.5 mg/g, respectively). However, in the extraction of phenolic compounds from the berries the effect of the solvent was even more significant since the extraction with methanol was much higher than the extraction obtained when using water (29.7 mg/g and 3.1 mg/g, respectively). The extraction of phenolic compounds using methanol as the solvent showed to be more efficient for both berries and leaves of *C. album*.

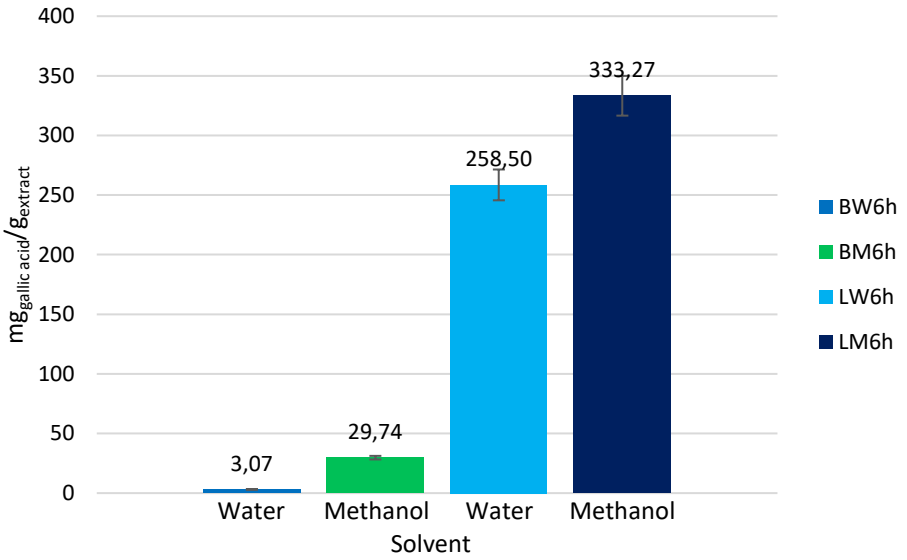

Figure 7 – Effect of the solvent used for the preparation of the extracts in the extraction of phenolic compounds from the *C. album* berries and leaves.

3.5. Determination of flavonoid compounds of the extracts

The flavonoid compounds content of lyophilized berries extracts and grinded leaves extracts in methanol and water was determined using the Al-Dabbas *et al.* (2006) method[104] with slight modifications. The graphic with the calibration curve of the quercetin solution is present in Figure 8. For the determination of flavonoids only a quercetin calibration curve with methanol as solvent was made since in the previous experiment of determination of total phenolic compounds was possible to verify that the solvent used (water or methanol) does not have a great influence on the calibration curve.

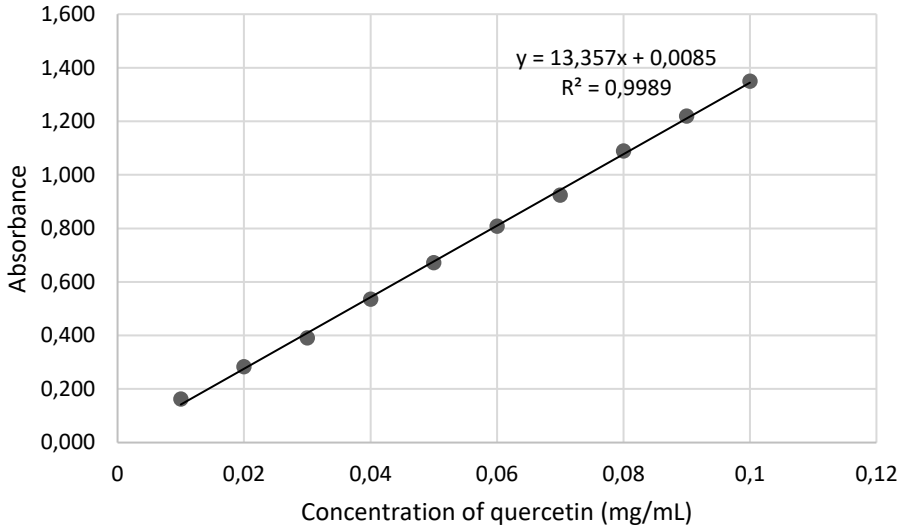

Figure 8 – Standard curve of methanolic quercetin solution.

In the same way as for phenolic compounds, using the equation of the standard curve was possible to determine the equivalent amount of quercetin present in each extract in mg/mL. By multiplying this value by the inverse of the extract concentration was obtained the concentration of quercetin in mg per mg of extract. The flavonoid compounds present in the extracts are expressed as mg of quercetin equivalents per g of extract and the results are shown on Table 6.

Table 6 – Total flavonoid compounds content of the *C. album* extracts expressed in milligrams of quercetin equivalents per gram of extract (mg/g).

Extracts	**Quercetin equivalents per gram of extract (mg/g)**
BW6h	4.2
BM6h	5.6
LW16h	52.6
LW6h	35.9
LM6h	107.5

As has been shown for phenolic compounds, and also for both the solvents used in the extraction (water and methanol) and comparing the same time of extraction (6 h), the amount of flavonoids in mg per g of extract is much higher in the leaves (35.9 mg/g in the aqueous extract and 107.5 mg/g in the methanol

extract) than in the berries (4.2 mg/g in the aqueous extract and 5.6 mg/g in the methanol extract). This result suggests that *C. album* leaves extract also has a higher potential as a source of flavonoids than the berries extract.

However, in the case of flavonoids, the amount of flavonoids present in the other water leaves extract showed that the attempt to make a leaf extract in water for a longer time (16 h) resulted in an increase of the amount of flavonoids extracted. This is possible to verify when comparing the extract of leaves in water with a time of extraction of 16 h (52.6 mg/g) with the other leaves extract also in water but with a time of extraction of 6 h (35.9 mg/g). In this case the increase may suggest that the extraction of the leaves with water over a long period of time was not accompanied by the degradation of flavonoids, even though some phenolic compounds may have been degraded.

The effect of the solvent used for the preparation of the extracts on the extraction of flavonoids from the berries and leaves of *C. album* is present in Figure 9, comparing only the extracts obtained with the same extraction time (6 h). The results suggest that the solvent used has an effect on the extraction of the flavonoids present, from both berries and leaves. For the leaves the effect of the solvent was even more significant since the extraction of the flavonoids with methanol is much higher than that achieved with water (107.5 mg/g and 35.9 mg/g, respectively). In the extraction of flavonoids from the berries the solvent has a reduced effect since the values obtained are similar (5.6 mg/g with methanol and 4.2 mg/g with water). The extraction of flavonoids using methanol as the solvent also showed to be more efficient for both berries and leaves of *C. album*.

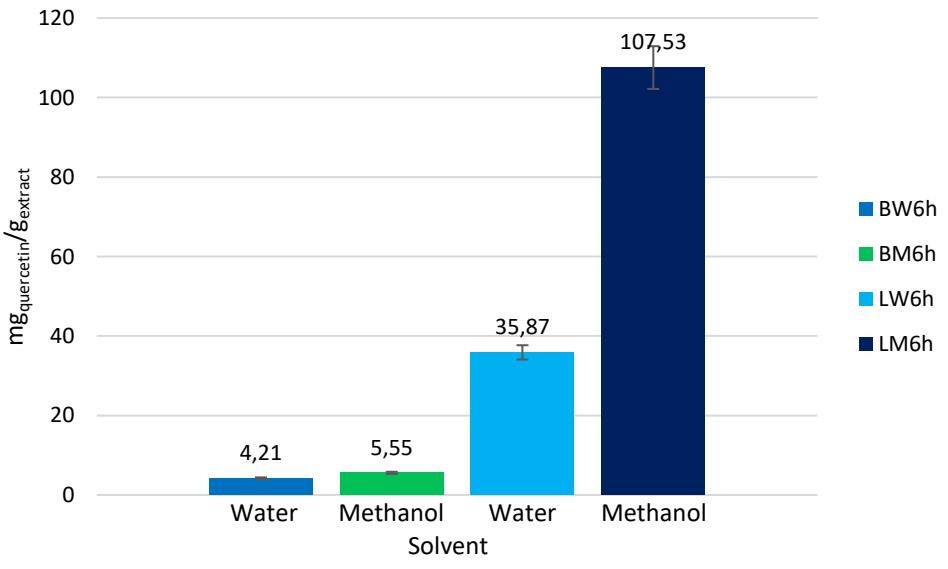

Figure 9 – Effect of the solvent used for the preparation of the extracts in the extraction of flavonoid compounds from the *C. album* berries and leaves.

3.6. Determination of antioxidant activity of the extracts

To determine the potential antioxidant activity of the extracts of berries and leaves of *C. album* was used the DPPH free radical scavenging method. The calculated antioxidant activity with IC_{50} values for each one of the five extracts is shown in Table 7.

Table 7 – The IC_{50} values of DPPH scavenging effect of *C. album* berries and leaves extracts.

Extract	**Extract concentration for IC_{50} (μg/mL)**
BW6h	8.7
BM6h	9.3
LW16h	7.7
LW6h	7.5
LM6h	8.6

In this experiment both berries and leaves extracts showed to have antioxidant activity. The results suggest that the leaves extracts (IC_{50} of 7.5 μg/mL in water and 8.6 μg/mL in methanol) have a higher antioxidant capacity than the berries extracts (IC_{50} of 8.7 μg/mL in water and 9.3 μg/mL in methanol) by having lower IC_{50} values, meaning that a lower concentration of leaf extract is needed to capture 50% of DPPH free radicals, and this result is valid for both solvents used. However, this difference is very small, considering that the values are expressed in μg/mL, and may not necessarily mean that the leaves extracts of *Corema album* are more abundant in antioxidants than the berries extracts.

Also, a longer leaf extraction time (16 h) did not result in an increase in antioxidant activity (IC_{50} of 7.7 μg/mL with 16 h of extraction and 7.5 μg/mL with 6 h of extraction), which may mean that the extraction over a long period of time does not lead to more antioxidants being extracted.

The effect of the solvent used for the preparation of the extracts on the antioxidant activity of the extracts of berries and leaves of *C. album* is present in Figure 10. The results suggest that the solvent used may have an effect on the IC_{50} values for both berries and leaves. In this case, the extraction with water may lead to the extraction of more antioxidants from both berries and leaves (for berries the IC_{50} is 8.7 μg/mL in water and 9.3 μg/mL in methanol and for leaves the IC_{50} is 7.5 μg/mL in water and 8.6 μg/mL in methanol). However, considering that the IC_{50} values are expressed in μg/mL, this difference is small, and may not

necessarily mean that water is a better solvent for extracting antioxidants from berries and leaves of *C. album*.

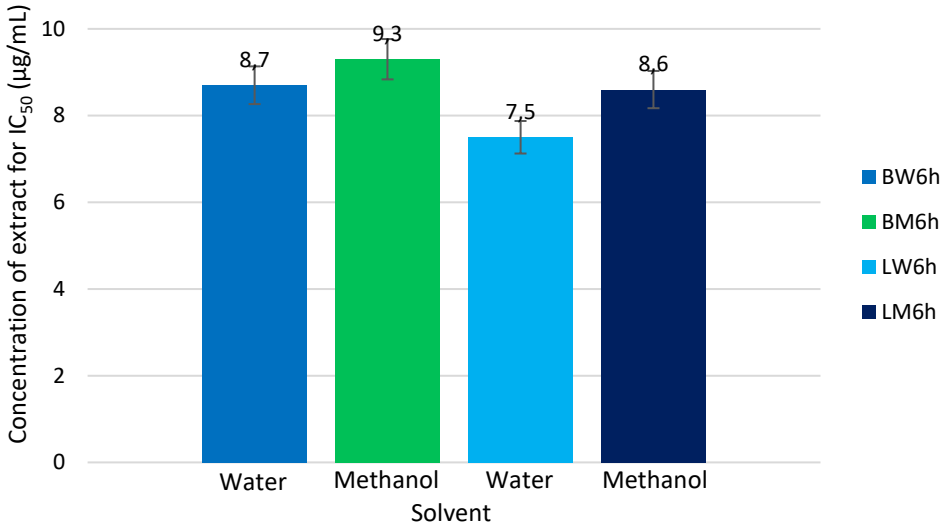

Figure 10 – Effect of the solvent used in the IC_{50} values of DPPH scavenging effect of *C. album* berries and leaves extracts.

3.7. Identification and quantification of phenolic compounds by HPLC

The area of the peaks is present in Table 8 and the concentration in the extracts of berries and leaves of *C. album* of trigonelline, caffeic acid, p-coumaric acid, ferulic acid, 3-CQA, 4-CQA and 5-CQA is present in Table 9. The values are the average of duplicate determinations. For the alkaloid trigonelline and the phenolic acids (hydroxycinnamic acids) caffeic acid, p-coumaric acid and ferulic acid the equation of the standard curve and the respective peak area were used to determine the concentration of each compound in mg of compound per mL of extract. By multiplying this value by the inverse of the extract concentration was obtained the concentration of compound in mg per 100 mg of extract. In the case of the polyphenolic compounds 3-CQA, 4-CQA and 5-CQA the quantification of the isomers in the samples was achieved by comparison of peak areas with a standard of 5-caffeoylquinic acid (with peak area of 37770800 mAU), allowing for differences in molar absorptivity between the isomer in question and the standard. For this experiment the $RF_{5\text{-CQA standard}} = 1.324\text{x}10^{-8}$ mg/mL/mAU; $\varepsilon_{3\text{-CQA}} = 18400$; $\varepsilon_{4\text{-CQA}} = 18000$; $\varepsilon_{5\text{-CQA}} = 19500$ and $M_{r1} = M_{r2} = 354.31$. After determining the peak areas was possible to determine the concentration of each isomer in mg of

isomer per mL of extract, and in the same way as for the other compounds, multiplication of this value by the inverse of the extract concentration gave the concentration of compound in mg per 100 mg of extract. All the concentrations shown are expressed in terms of wet matter.

Table 8 – Average peak areas obtained by HPLC for the phenolic compounds tested in the berries and leaves extracts. Chromatographs were acquired at λ_{max} of 272 nm and 320 nm, depending on the compounds.

	Peak area (mAU)						
	272 nm	**320 nm**					
Extract	**Trigonelline**	**Caffeic acid**	**p-coumaric acid**	**Ferulic acid**	**3-CQA**	**4-CQA**	**5-CQA**
Lyophilized berries powder	156199	3319979	0	0	2626667	1025695	20974732
Grinded leaves	722157	0	0	323825	247379	449442	9420548

Table 9 – Concentration of the phenolic compounds tested in the berries and leaves extracts expressed in μg of compound per 100 mg of extract for wet matter.

	Concentration (μg/100 mg in wet matter)						
	272 nm	**320 nm**					
Extract	**Trigonelline**	**Caffeic acid**	**p-coumaric acid**	**Ferulic acid**	**3-CQA**	**4-CQA**	**5-CQA**
Lyophilized berries powder	2.5	10.1	0.0	0.0	15.0	6.0	112.9
Grinded leaves	10.2	0.0	0.0	0.9	1.2	2.3	43.0

The results showed that both lyophilized berries powder and grinded leaves extracts have the alkaloid trigonelline and the isomeric polyphenols 3-CQA, 4-CQA and 5-CQA and also any of the extracts has the phenolic acid p-coumaric acid. In relation to the other two phenolic acids, the berries extract contains caffeic acid, but the leaves extract did not show to have this compound. The opposite was verified for the ferulic acid, which is present in the leaves but was not found in the berries.

From the results it was also possible to verify that the leaves extract is richer in the alkaloid trigonelline (10.2 μg/100 mg) than the berries extract (2.5 μg/100 mg) but the berries extract is richer in the polyphenol chlorogenic acid and its two isomers studied, 4-CQA and 5-CQA (15.0 μg/100 mg, 6.0 μg/100 mg and 112.9 μg/100 mg, respectively), than the leaves extract (1.2 μg/100 mg, 2.3 μg/100 mg and 43.0 μg/100 mg, respectively). Therefore, the berries extract is a

better source of caffeic acid, chlorogenic acid and its isomers, 4-CQA and 5-CQA, and the leaves extract is a better source of trigonelline and ferulic acid.

3.8. Infrared spectroscopy: attenuated total reflectance measurements

To study the composition of the samples of berries (lyophilized berries powder) and leaves (grinded leaves) of *C. album* an ATR-FTIR spectroscopy was performed. As the berries powder contains pieces of the skin, pulp and seeds of the berries a comparison of the spectrum obtained with the spectra already available in the laboratory (Luís Simões) of the skin, pulp and seeds was made. At the present level of understanding it was not possible to identify all of the specific compounds responsible for every spectral feature. However, it was possible to identify major classes of compounds in the lyophilized berries powder and grinded leaves samples and, in the case of the berries sample, to recognize whether a particular peak or shoulder is due to the presence of skin, pulp or seeds in the sample.

3.8.1. ATR-FTIR spectral features of berries

The ATR-FTIR spectrum of lyophilized berries powder of *C. album*, in the 4000-400 cm^{-1} spectral region, is present in appendix (Figure 2A). The most prominent spectral profile, which spreads across the 3700-3000 cm^{-1} region, is correlated with the O-H elongation (ν(OH)). Its intensity denotes the presence of water and other biochemical constituents that also contribute to this spectral interval, such as polysaccharides, phenolic compounds and non-esterified hydroxyl groups[127,128]. Bands appearing in the region of 2917-2916 cm^{-1} and 2850-2849 cm^{-1} were assigned to the asymmetric (ν_{as}) and symmetric (ν_s) CH_2 stretching vibrations, $\nu_{as}(CH_2)$ and $\nu_s(CH_2)$ respectively[129]. A week shoulder at 2890 cm^{-1} corresponds to C-H stretching (ν(CH))[128]. The presence of carbonyl-containing compounds in the sample is reflected in the relatively broad band with an absorbance maximum at 1717 cm^{-1}. This spectral feature is assigned to the C=O stretching mode (ν(C=O))[127,129]. Immediately following this band, on the lower frequency side, appears another band with a maximum of absorption at 1638 cm^{-1}. Part of the considerable intensity absorption of this band may be due to the contribution of the deformation mode H–O–H of water ($\delta(H_2O)$)[128].

The following bands bellow 1500 cm^{-1} are in the so-called "fingerprint region" (1500-600 cm^{-1}). At these frequencies quite similar molecules give different absorption patterns. The peaks in this region are known to be associated with C-O and C-C stretches in sugars, esters and organic acids, amongst other compounds[128,130]. High intensity peaks in the 1300-1000 cm^{-1} spectral region may be due to the phenolic compounds present in the berries[128]. By comparing the spectrum of the berries powder sample to the spectra of the skin, pulp and seeds of white crowberry these peaks were mainly attributed to the phenols present in the seeds (in appendix, Figure 3A). It was possible to verify that the peak at 1341 cm^{-1} and the shoulder at 977 cm^{-1} were both due to the presence of seeds in the sample. Also, the sequence of peaks in the 935-750 cm^{-1} spectral region (peaks at 917, 896, 866, 817 and 776 cm^{-1}) were associated to the presence of seeds[131,132].

3.8.2. ATR-FTIR spectral features of leaves

The ATR-FTIR spectrum of fresh grinded leaves of *C. album*, in the 4000-400 cm^{-1} spectral region, is present in appendix (Figure 4A). The most prominent spectral profile, which spreads across the 3700-3000 cm^{-1} region, is correlated with the O-H elongation (ν(OH)). Its intensity denotes the presence of water, which was expected as leaves have a high water content. Other biochemical constituents that may be also contributing to this spectral interval are polysaccharides, phenolic compounds and non-esterified hydroxyl groups, for example[127,128]. Two strong bands at approximately 2918 and 2850 cm^{-1} were assigned to the asymmetrical and symmetrical stretching vibrations of CH_2 groups, $\nu_{as}(CH_2)$ and $\nu_s(CH_2)$ respectively. These bands are accompanied by the corresponding $\delta(CH_2)$ bending vibrations at around 1467, 1313, and 725 cm^{-1}[127]. The presence of carbonyl-containing compounds in the sample is reflected in the bands with an absorbance maximum at 1731 cm^{-1} (and its shoulder at 1715 cm^{-1}) and 1686 cm^{-1}. This spectral feature was assigned to the C=O stretching mode (ν(C=O)) of the cuticle constituents of the plant, such as esters and carboxylic acids, mainly due to the presence of cutin (main component of the plant cuticle)[127,129]. Immediately following this band, on the lower frequency side, appears another band with a shoulder at 1638 cm^{-1}. Part of the considerable intensity absorption of this band may be due to the contribution of the deformation mode H–O–H of water ($\delta(H_2O)$)[128].

The following bands bellow 1500 cm^{-1} are in the so-called "fingerprint region" (1500-600 cm^{-1}). At these frequencies quite similar molecules give different

absorption patterns[128]. Cellulose is mainly characterized by two strong bands at 1055 cm^{-1} and 1032 cm^{-1} [119]. These frequencies are well visible in the spectrum, with a broad band around 1032 cm^{-1} and a shoulder at approximately 1055 cm^{-1}.

3.9. Cell studies

3.9.1. Cell viability measured by MTT assay

The results obtained for the cytotoxic effect of the BW6h and LW6h extracts in the reduction of cell viability evaluated on HT-29 cancer cell line are shown in Figure 11.

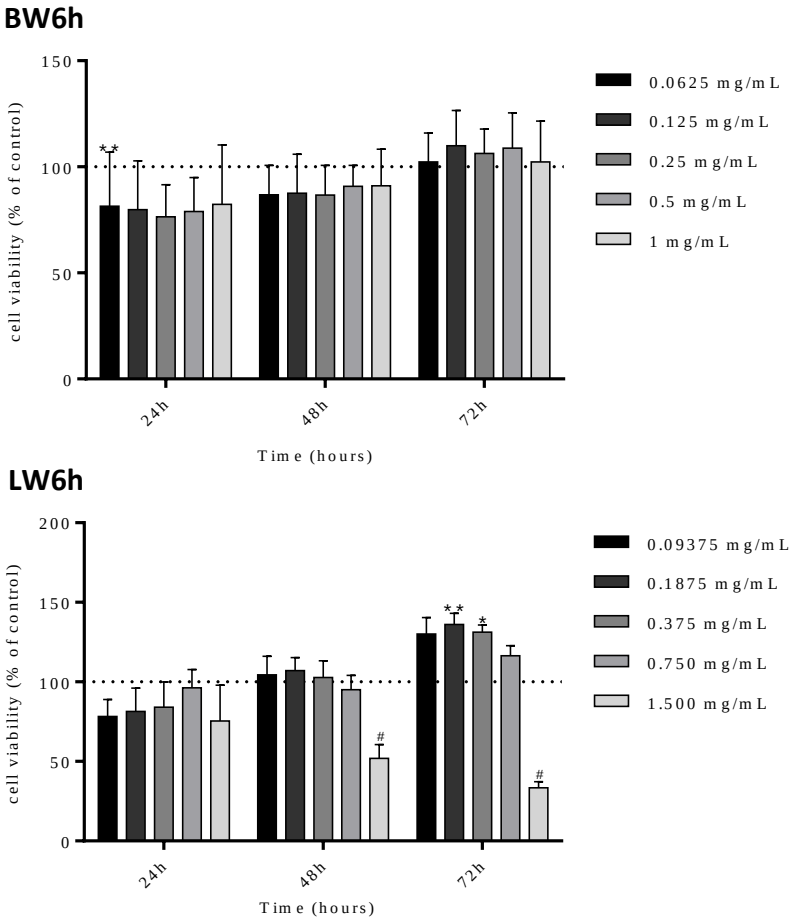

Figure 11 – Cytotoxic effect of *C. album* BW6h and LW6h extracts. The results are presented in percentage relative to control cultures taken as 100% (horizontal dashed line). All cultures were plated with cell density $3.0x10^4$ cells/cm^2. The values presented are the mean ± SD of the values obtained for three independent assays, each one performed in triplicate. Statistical analysis was performed using the One-Way ANOVA, followed by Turkey's multiple comparisons test. Differences were considered statistically significant with: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ or # $p < 0.0001$.

For LW6h extract it was possible to verify that the effect depends on the concentration administered and the incubation time (24, 48 and 72 h). For BW6h extract the effect was time dependent but only slightly concentration dependent. Leaf extract induced cell death, resulting in a reduction of cell viability between 60 and 70%, at a concentration of 1.5 mg/mL, after 72 h. The induction of cell death began to be verified after 24 h of incubation (between 20 and 30%), continued to increase after 48 h of exposure (between 40 and 50%) and reached the maximum after 72 h (between 60 and 70%), for the times tested. In the case of the berries extract there was no cytotoxic effect verified, both in terms of the concentrations applied (1.00, 0.50, 0.25, 0.13 and 0.06 mg/mL) and the incubation time (24, 48 and 72 h), being no dose-response relationship for exposure of the HT-29 cell line to this plant extract, in the range of concentrations tested. After 24 h of application of the BW6h extract, there was a decrease in cell viability between 10 and 30% for all concentrations tested, however the cells showed signs of recovery after 48 h of incubation, and fully recovered their viability after 72 h.

The IC_{50} values, concentrations required to decrease cell viability by 50%, were calculated, when applicable, and are shown in table 10, for all the exposure times tested. The results showed that there was a great variability of the LW6h extract effect in the HT-29 cancer cells at 72 h, which could be verified by the high SD value calculated, so the result is not shown. The leaves extract had cytotoxic effect on the cell line tested, being more effective after 48 h of exposure of the cells to the extract in water since the concentration required to reduce the cell viability in 50% was the lowest (IC_{50} value of 9.212×10^{-2} mg/mL). For the berries a decrease in cell viability was not achieved by 50% for any of the times tested.

Table 10 – IC_{50} values (mg/mL) for the *C. album* BW6h and LW6h extracts tested, obtained with the MTT assay after 24, 48 and 72 h of incubation.

IC_{50} (mg/mL)			
Time (h) / **Extract**	**24**	**48**	**72**
BW6h	n. a.	n. a.	n. a.
LW6h	6139	9.212×10^{-2}	-

n.a. - not applicable (was not reached a reduction of 50% in cell viability)

3.9.2. Cell proliferation measured by SRB assay

The results obtained for the effect on the cell density of the BW6h and LW6h extracts are shown in Figure 12.

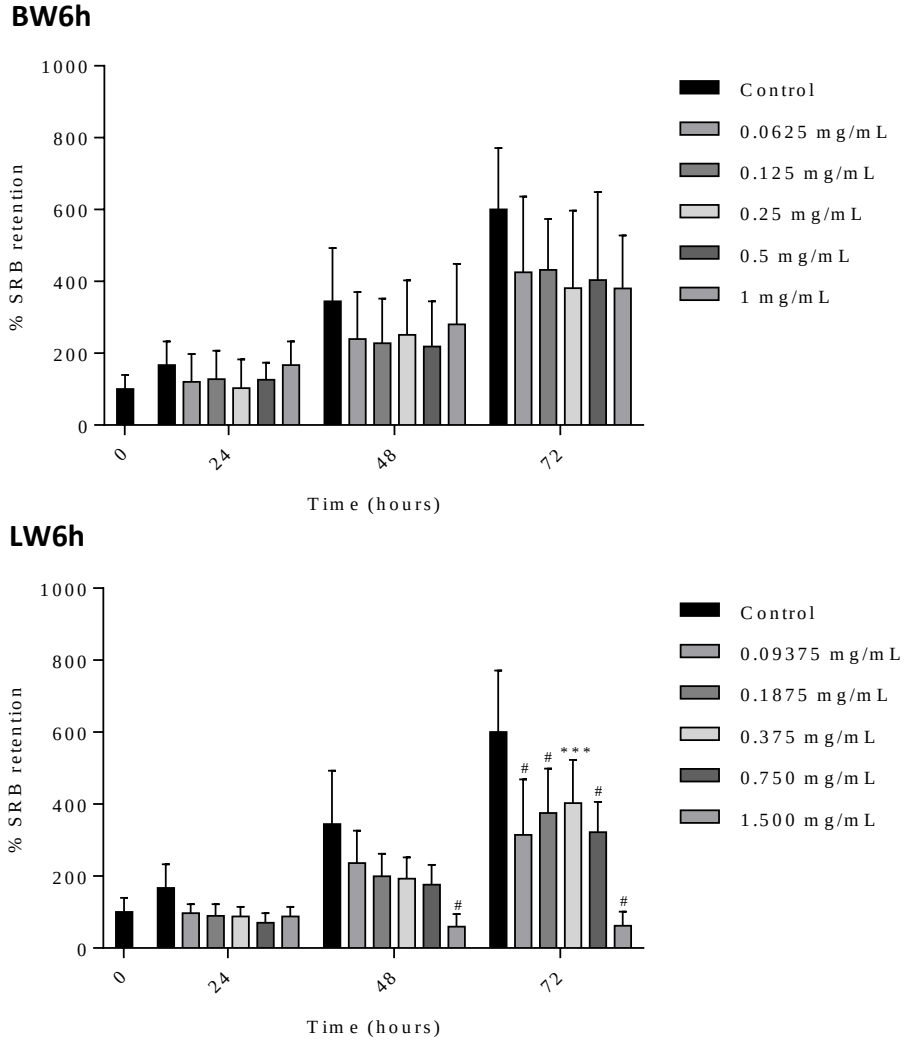

Figure 12 – Cell proliferation in the presence of *C. album* BW6h and LW6h extracts. The results are presented in % of SRB retention in relation to control cell cultures at time 0 h (C_0). All cultures were plated with cell density $3.0x10^4$ cells/cm^2. The values presented are the mean ± SD of the values obtained for three independent assays, each one performed in triplicate. Statistical analysis was performed using the One-Way ANOVA, followed by Turkey's multiple comparisons test. Differences were considered statistically significant with: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ or # $p < 0.0001$.

From the results shown in the graphs it is possible to verify a decrease in cell density over time and consequently a decrease in cellular proliferation, in the cells in which the leaves extract was applied at the highest concentration tested, 1.5 mg/mL, corroborating the MTT results for this concentration. For the other

leaves extract concentrations tested was also verified a decrease in cell density at all times tested, by comparison with the control at each time, but the cells continued to be able to proliferate. In the case of the berries extract a decrease in cell density was also verified for all the concentrations tested and at all the times tested, by comparison with the control at each time, but the cells continued to be able to proliferate.

The IC_{50} values of this assay were calculated, when applicable, and the values are shown in Table 11, for all the exposure times tested. The LW6h extract lead to a decrease in cell density by 50% at all times, with the IC_{50} value of 2.339×10^{-6} mg/mL at 24h, 6.667×10^{-4} mg/mL at 48 h and 1.237×10^{-2} mg/mL at 72 h. For the BW6h extract a decrease in cell density was only achieved by 50% after 24 h of incubation (IC_{50} value of 1.629×10^{-2} mg/mL).

Table 11 – IC_{50} values (mg/mL) for the *C. album* BW6h and LW6h extracts tested, obtained with the SRB assay after 24, 48 and 72 h of incubation.

IC_{50} (mg/mL)			
Time (h) / **Extract**	**24**	**48**	**72**
BW6h	1.629×10^{-2}	n. a.	n. a.
LW6h	2.339×10^{-6}	6.667×10^{-4}	1.237×10^{-2}

n.a. - not applicable (was not reached a reduction of 50% in cell viability)

3.10. Sensory profile of cookies and bread with white crowberries

3.10.1. Final products

The cookies used for sensory analysis contained 3 g of white crowberries powder per 260 g of cookie dough because these were the cookies considered by us as having a more pleasant taste, without being too acidic. They are shown in Figure 13. The cookies made following the same recipe but without the addition of berries (standard cookies) are also shown in Figure 13. For the bread, the one with the taste considered more pleasant by us, with a quantity of berries that we considered adequate and without changing much the consistency after baking in

the oven (since the berries contain a lot of water, greatly increasing the humidity of the bread) was the one that contained 30% of whole berries, having been the bread chosen for sensory analysis. This bread was also more visually appealing because we could see the berries in the whole and cut bread. In the breads made with 20, 30 and 40% of crushed berries it was only possible to start to detect some flavour from the addition of 40% of berries, being necessary to use more berries for each bread and resulting in a large increase of humidity, making it difficult to bake and changing its final consistency. The white crowberry bread and the standard bread, made following the same recipe but without the addition of berries, are shown in Figure 14.

Figure 13 – White crowberry cookies made with white crowberries powder (1) and standard butter cookies (2).

Figure 14 – White crowberry bread made with whole berries (1) and standard bread (2).

3.10.2. Consumer acceptability and intention to buy of white crowberry cookies

The cookies sensory panel was composed of 40 panelists, 72.5% were women and 27.5% were men. The mean age was 29.6 ± 14.9 and 72.5% of the panelists were less than 25 years old, 0.0% were 26-35 years old, 10.0% were 36-45 years old and 17.5% were more than 45 years old. The global appreciation of white crowberry cookies was quite positive (7.2 ± 1.3) and very close to the global appreciation of butter cookies (7.3 ± 1.0), as it is possible to see in Figure 15. All the sensory characteristics (visual aspect, colour, smell, taste and texture) of white crowberry cookies were very positive (7.1 ± 1.4; 7.1 ± 1.3; 6.8 ± 1.6; 7.2 ± 1.7 and 6.8 ± 1.5, respectively) and close to the values given to butter cookies (7.5 ± 1.1; 7.4 ± 1.0; 7.6 ± 1.2; 7.1 ± 1.2 and 6.5 ± 1.7, respectively). The smell was the only sensory characteristic that greatly differed in the consumer acceptability profile of the white crowberry cookies, which may be due to the intense smell of the crowberries powder. Several of the panelists referred that the acidic flavour of the berries turned the cookies tasty, pleasant and unique.

The majority of the panelists showed intention to buy the white crowberry cookies (Figure 16), with 27.5% choosing 5 ("certainly would buy") and 40.0% choosing 4 ("probably would buy"). 22.5% of the panelists chose 3 ("maybe would buy, maybe would not buy"), only 10.0% chose 2 ("probably would not buy") and none of the respondents choose 1 ("certainly would not buy"). When compared to the results of the butter cookies, the respondents were more certain in their intention to buy the white crowberry cookies because only 12.5% choose 5 for the butter cookies (and 27.5% choose 5 for the white crowberry cookies),

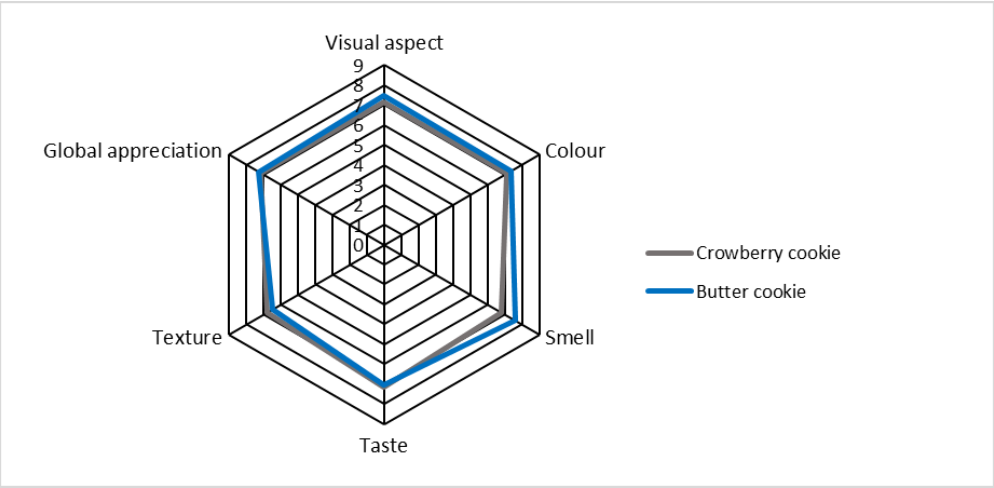

Figure 15 – Mean consumer acceptability of white crowberry cookies and butter cookies attributed by the 40 panelists. Sensory characteristics were rated on a 9-point scale from 1 – really dislike, to 9 – really like.

and more respondents chose 4 (62.5%). 17.5% chose 3, 2.5% chose 2 and 5.0% of the panelists chose 1.

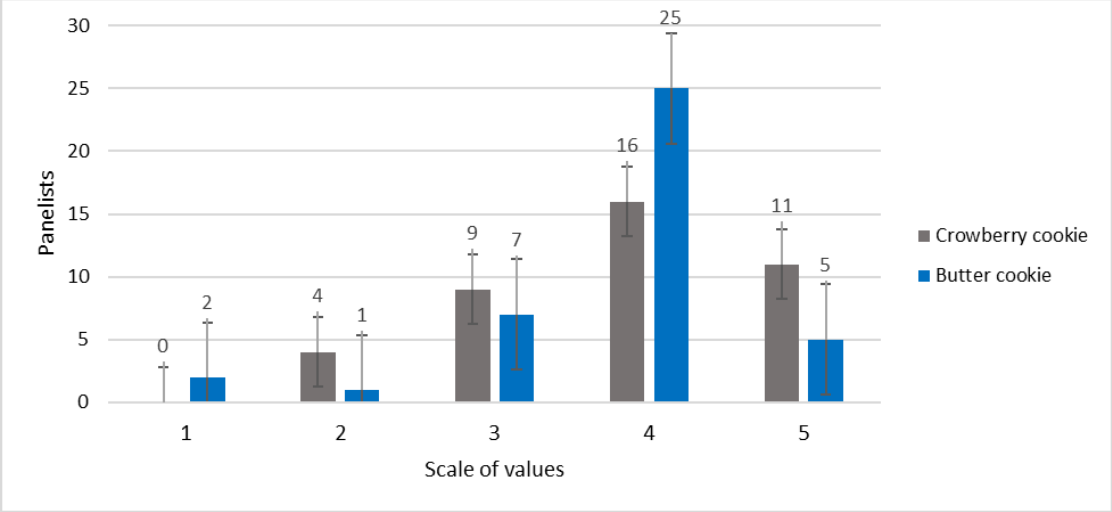

Figure 16 – Mean consumer intention to buy of white crowberry cookies and butter cookies attributed by the 40 panelists. Sensory characteristics were rated on a 5-point scale from 1 – certainly would not buy, to 5 – certainly would buy.

3.10.3. Consumer acceptability and intention to buy of white crowberry bread

The bread sensory panel was composed of 38 panelists, 71.1% were women and 28.9% were men. The mean age was 29.6 ± 15.1 and 73.7% of the panelists were less than 25 years old, 0.0% were 26-35 years old, 7.9% were 36-45 years old and 18.4% were more than 45 years old. The global appreciation of white crowberry bread was positive (6.2 ± 1.6) and not far from the global appreciation of simple homemade bread (7.4 ± 1.1), as it is possible to see in Figure 17. All the sensory characteristics (visual aspect, colour, smell, taste and texture) of white crowberry bread were positive (6.4 ± 1.8; 6.8 ± 1.5; 6.4 ± 1.5; 6.1 ± 1.8 and 6.5 ± 2.0, respectively) and also not far from the values given to simple bread (7.4 ± 1.1; 7.4 ± 1.2; 7.1 ± 1.6; 7.1 ± 1.2 and 7.3 ± 1.3, respectively). Several of the panelists referred that the berries seeds were too hard to crack, even when they usually like bread with seeds. One possible way to improve the consumer acceptability of the white crowberry bread would be by removing the seeds from the berries, however the process would damage the shape of the berries and the original idea of developing a bread with whole white crowberry berries on it.

The majority of the panelists were not certain if they would buy the white crowberry bread (Figure 18), with 26.3% choosing 4 and 26.3% choosing 3. Only 15.8% of the respondents were certain that they would buy the bread and chose

5. Also 26.3% of the panelists chose 2 and 5.3% choose 1. By comparing these results with the results of the simple homemade bread, it was possible to verify that the panelists preferred to buy the traditional bread, with 39.5% choosing 5 and 36.8% choosing 4. 21.1% chose 3, only 2.6% chose 2 and none chose 1.

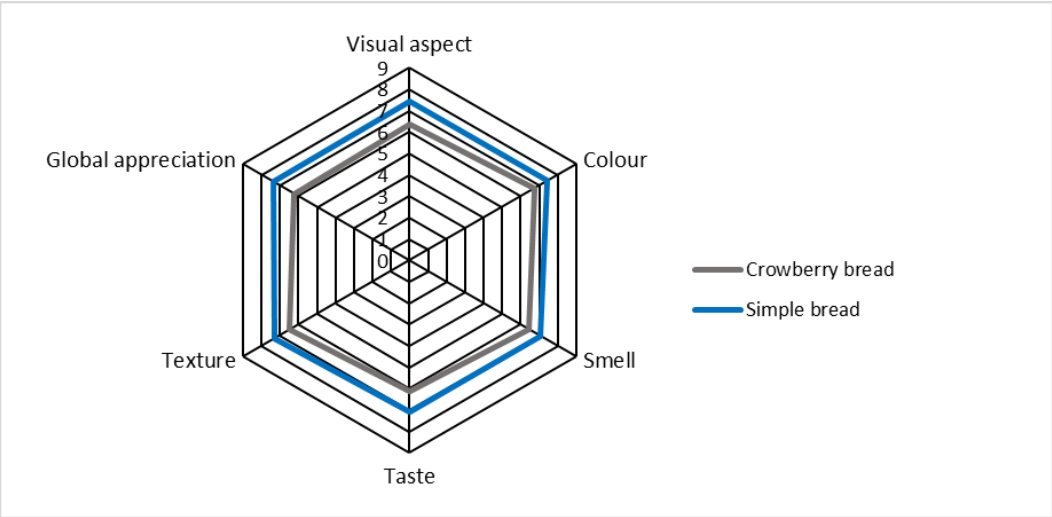

Figure 17 – Mean consumer acceptability of white crowberry bread and simple bread attributed by the 38 panelists. Sensory characteristics were rated on a 9-point scale from 1 – really dislike, to 9 – really like.

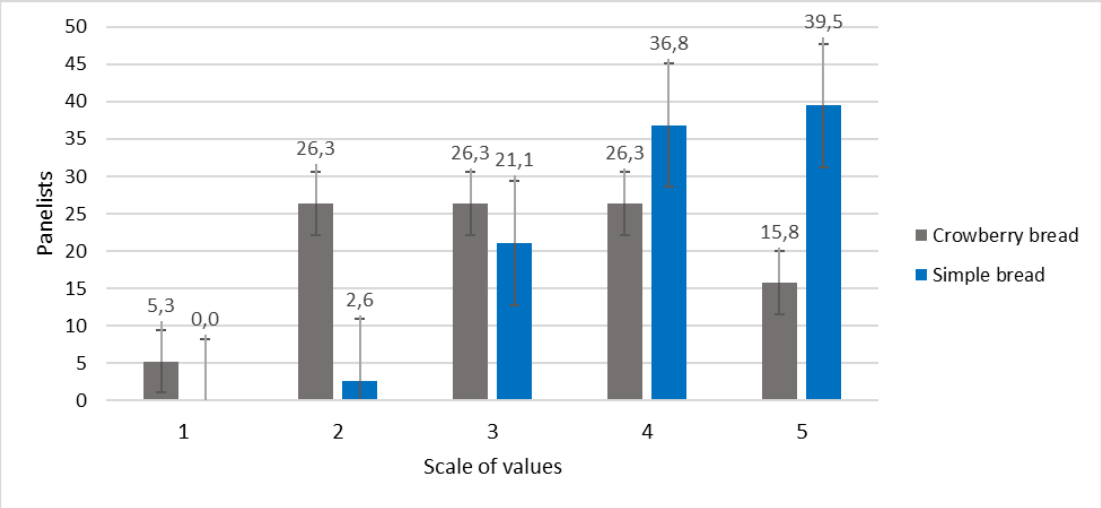

Figure 18 – Mean consumer intention to buy of white crowberry bread and simple bread attributed by the 38 panelists. Sensory characteristics were rated on a 5-point scale from 1 – certainly would not buy, to 5 – certainly would buy.

4

4. Conclusion

In this work was determined the nutrient profile of lyophilized berries powder of *C. album* and the phytochemical potential of the plant by quantification of total phenolic compounds content, in particular the flavonoids content, of aqueous and methanolic extracts made with its berries and leaves. The plant showed to be a source of phenolic compounds, and the results suggest that *C. album* leaves extracts have a higher potential as a source of total phenolic compounds and flavonoids than the berries extracts, for both solvents used in the extraction. However, the extraction of phenolic compounds and flavonoids using methanol as the solvent showed to be more efficient for both berries and leaves of *C. album*.

The antioxidant activity, measured by the DPPH free radical scavenging method, showed that both extracts of berries and leaves act as antioxidants in the presence of this free radical, being the antioxidant activity of the leaves extracts slightly superior. The results suggest that the solvent used may have an effect on the IC_{50} values for both berries and leaves and that the extraction with water may lead to the extraction of more antioxidants from both plant parts.

The identification and quantification of phenolic compounds by HPLC showed that both lyophilized berries powder and grinded leaves extracts had the alkaloid trigonelline and the isomeric polyphenols 3-CQA, 4-CQA and 5-CQA, and also any of the extracts had the phenolic acid p-coumaric acid. In relation to the other two phenolic acids identified and quantified, the berries extract contained caffeic acid, but the leaves extract did not show to have this compound

and the opposite was verified for the ferulic acid, which was present in the leaves but was not found in the berries. From the results it was also possible to verify that the berries extract is a better source of caffeic acid, chlorogenic acid and its isomers, 4-CQA and 5-CQA, and the leaves extract is a better source of trigonelline and ferulic acid.

With the ATR-FTIR spectroscopy results was possible to identify major classes of compounds in the lyophilized berries powder and grinded leaves samples and, in the case of the berries sample, to recognize whether a particular peak or shoulder was due to the presence of seeds in the sample. The peaks identified in the berries sample were correlated with the presence of water and other biochemical constituents that also contributed to that spectral interval, such as polysaccharides, phenolic compounds and non-esterified hydroxyl groups, and correlated to sugars, esters and organic acids, and were also present peaks that were associated with carbonyl-containing compounds. The peaks that were associated with the presence of phenolic compounds were mainly attributed to the presence of seeds in this sample. The peaks identified in the leaves sample were correlated with the presence of water, but there are other biochemical constituents that may be also contributing to that spectral interval, such as polysaccharides, phenolic compounds and non-esterified hydroxyl groups, and correlated to carbonyl-containing compounds and cuticle constituents of the plant, such as esters and carboxylic acids, mainly due to the presence of cutin (main component of the plant cuticle). Peaks associated with the presence of cellulose were also identified.

The leaves extract in water also had phytochemical potential as an anticancer agent, showing to have a cytotoxic effect in the studies performed with the HT-29 cancer cell line, inducing cell death, which was visible by a reduction of cell viability through the MTT assay and also by a reduction of cell proliferation through the SRB assay, when applied in a concentration of 1.5 mg/mL.

Foods were also developed with the addition of white crowberries, cookies and bread, which were generally well accepted by the sensory panel of non-trained panelists to which they were submitted for sensory evaluation, showing that the development of new food products may be a way to make these berries more known to the public and start being commercialized.

5

5. Future Work

As future work, the combined anticancer effect of the *C. album* leaves extract in water and drugs used in cancer treatment must be addressed in order to further clarify the possible use of this plant as a potential complementary treatment for cancer. Also, the possible leaf compounds involved in this effect need to be determined to enhance the effect that this plant can have against cancer through their extraction.

Another important achievement that needs to be done as future work is the determination of the compounds present in the plant's berries and leaves that are responsible for its antioxidant effect.

The cookies developed with the addition of white crowberries also need to be improved to increase their acceptance by consumers. One possible way to do this may be by adding stuffing made with berry jam.

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Appendix

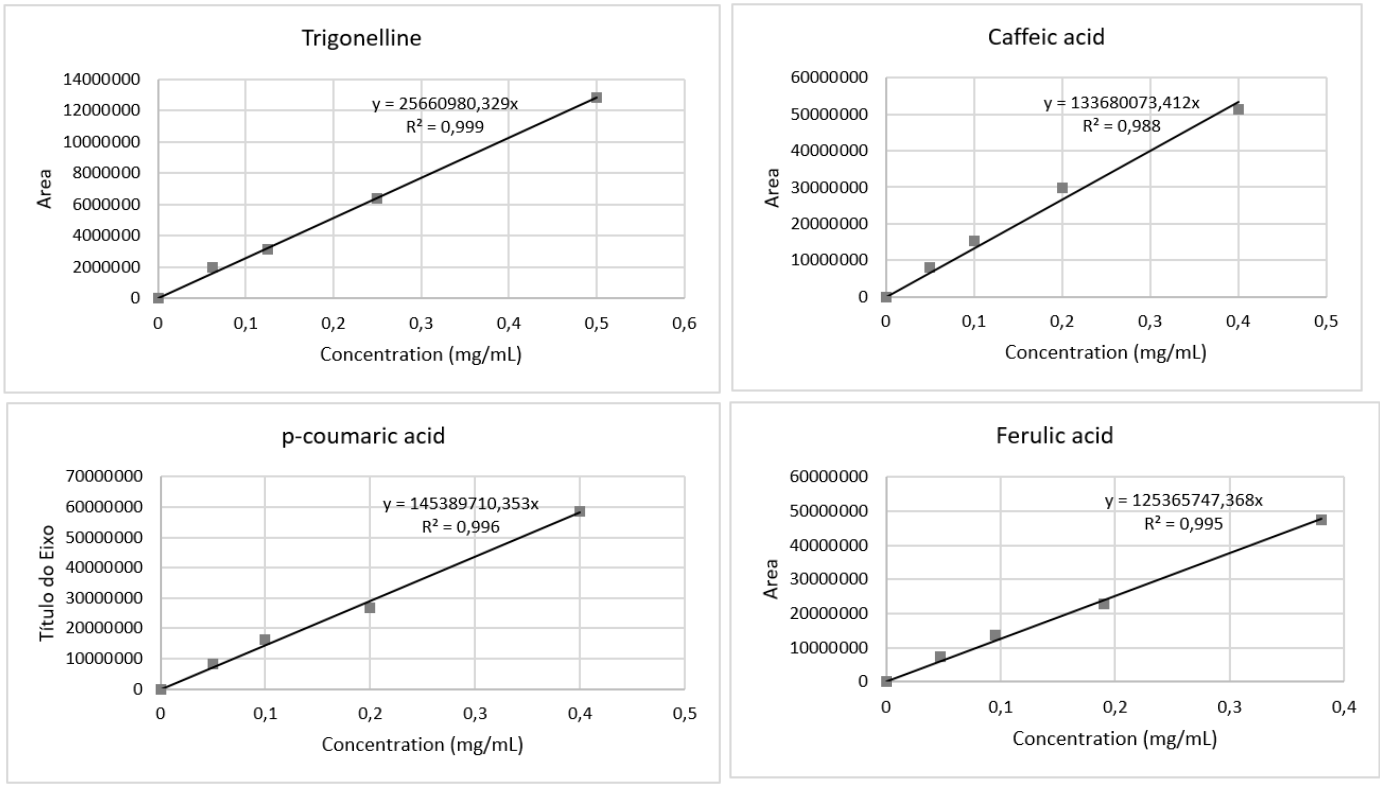

Figure 1A – Standard curves of trigonelline, caffeic acid, p-coumaric acid and ferulic acid obtained using different concentrations of each compound [A. Leitão, unpublished].

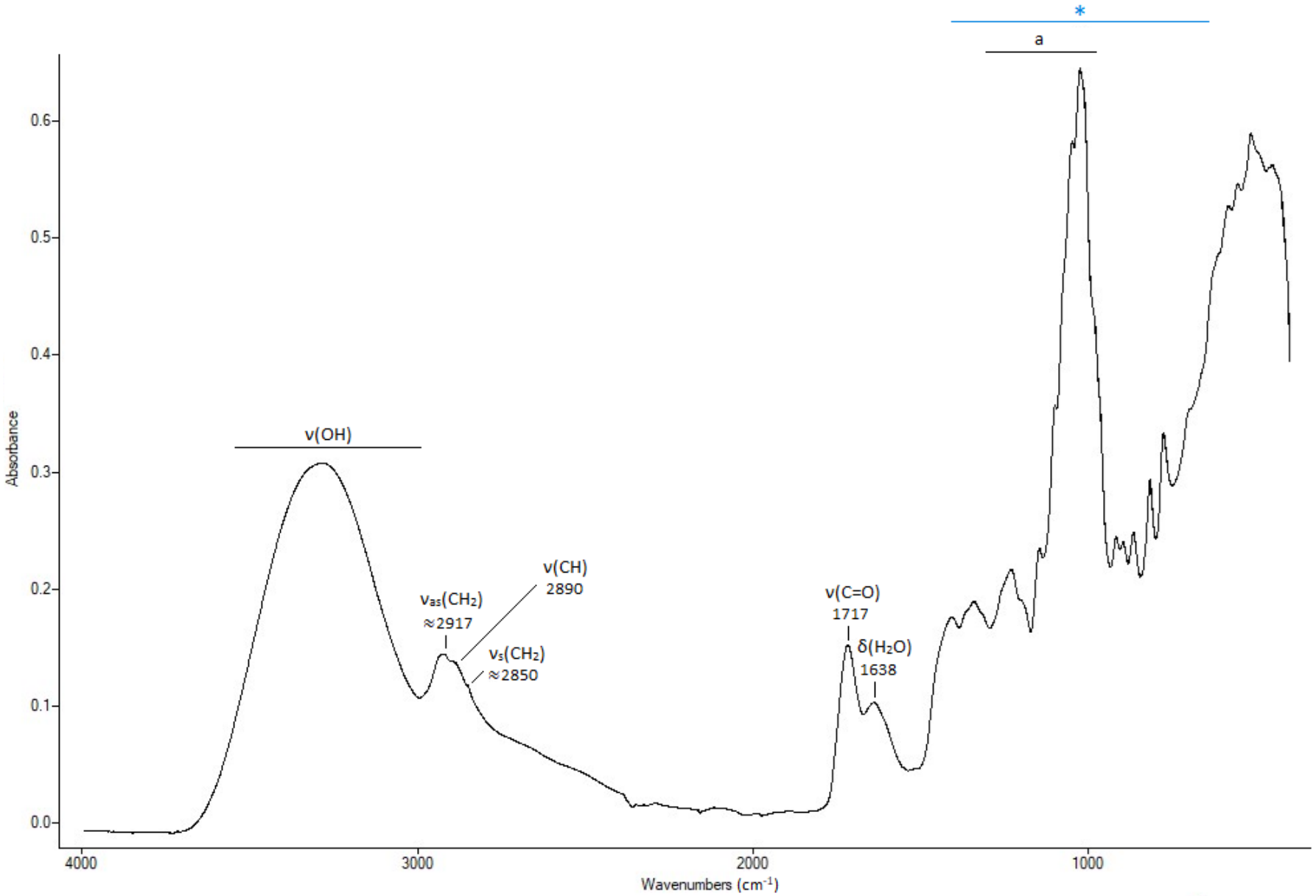

Figure 2A – ATR-FTIR spectrum of lyophilized berries powder of *C. album*. The identified bands are assigned. The spectral region marked with a (*) corresponds to the "fingerprint region" (1500-600 cm^{-1}) and the peaks in the region marked with a (a) were attributed to the phenolic compounds present in the berries (1300-1000 cm^{-1}).

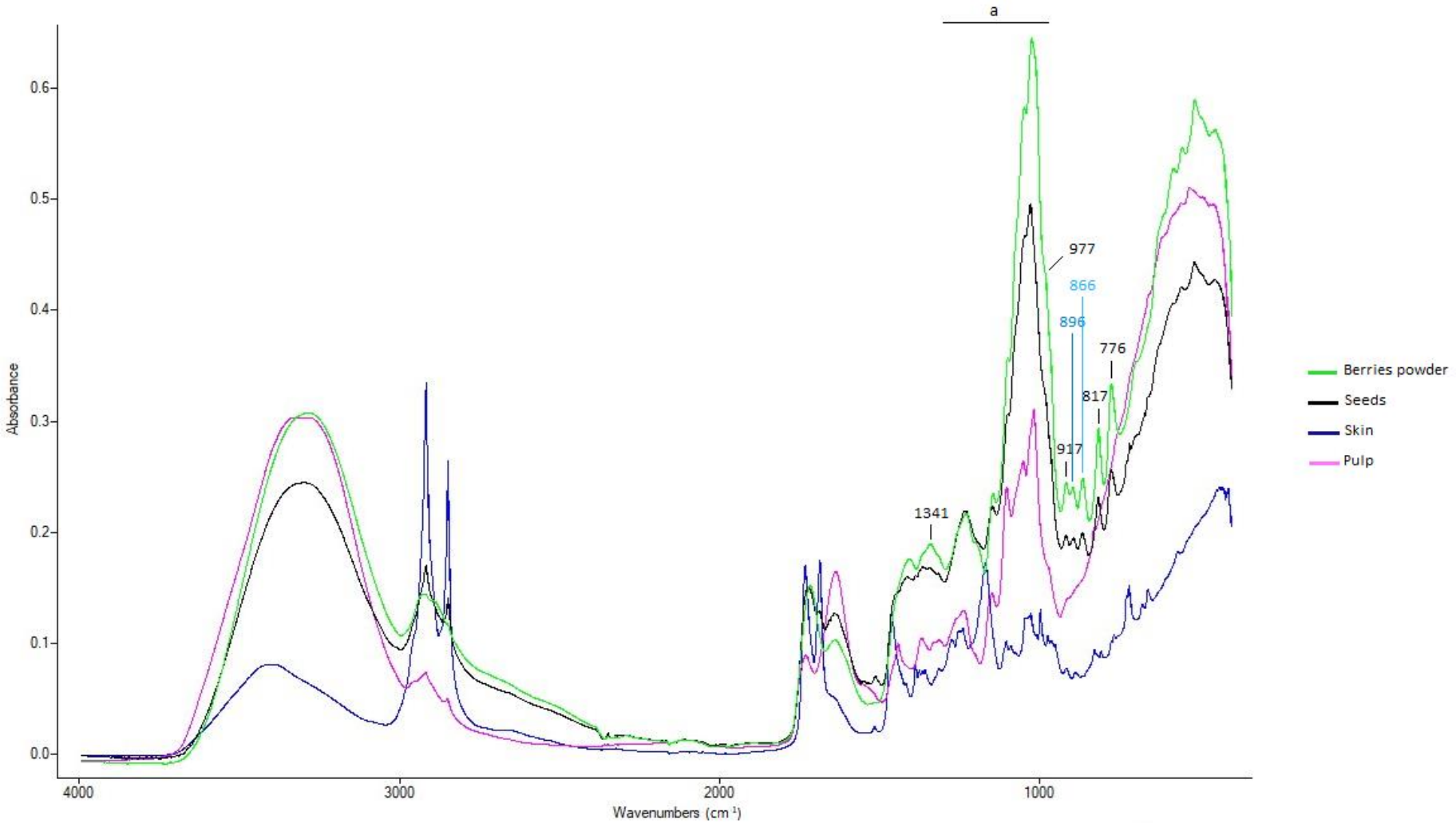

Figure 3A – ATR-FTIR spectra of lyophilized berries powder of *C. album,* skin, pulp and seeds. The identified peaks that are associated with the presence of seeds in the berries powder sample are assigned. The peaks in the region marked with a (a) were attributed to the phenolic compounds present in the berries (1300-1000 cm^{-1}).

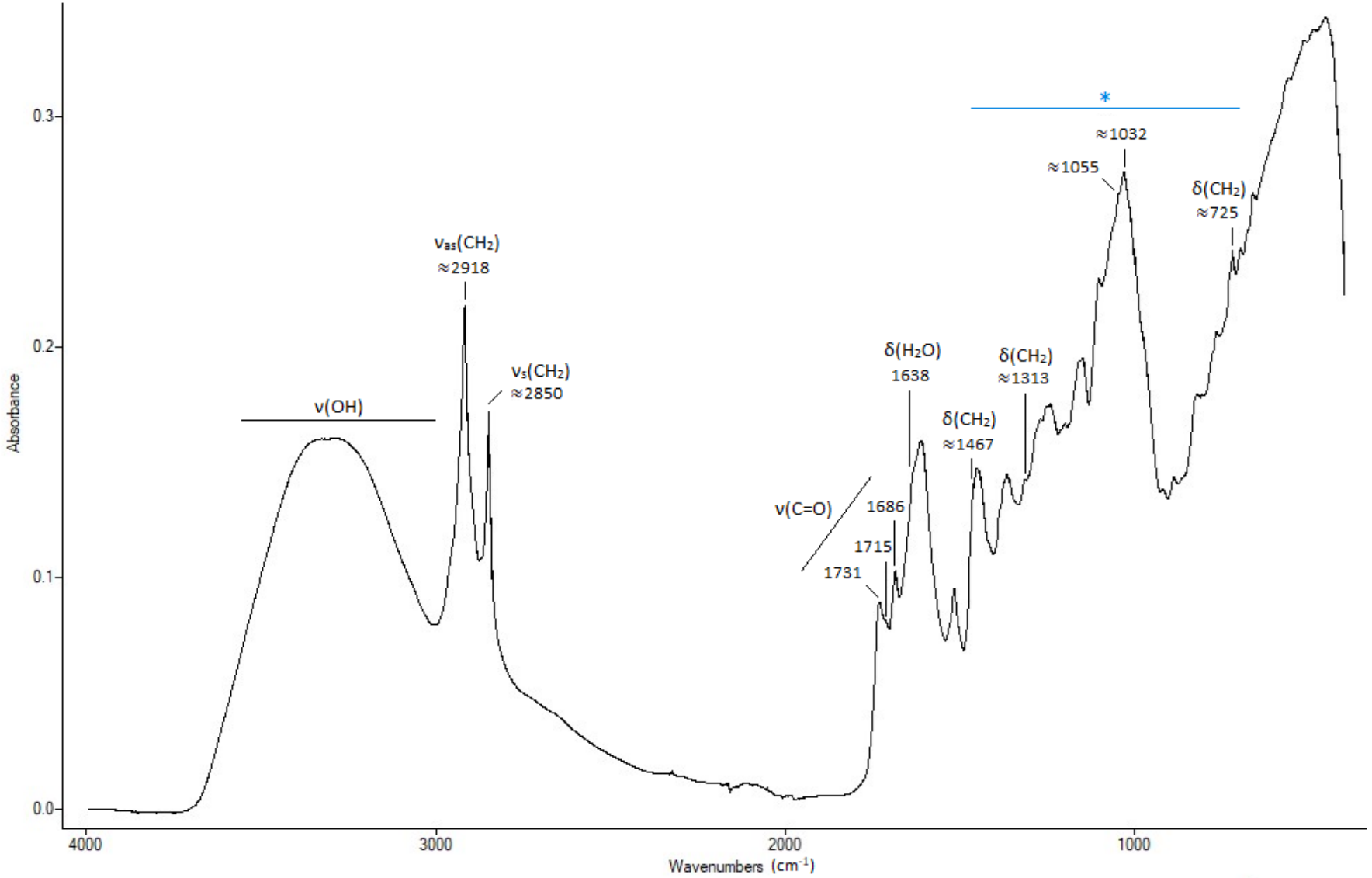

Figure 4A – ATR-FTIR spectrum of grinded leaves of *C. album*. The identified bands are assigned. The spectral region marked with a (*) corresponds to the "fingerprint region" (1500-600 cm^{-1}).