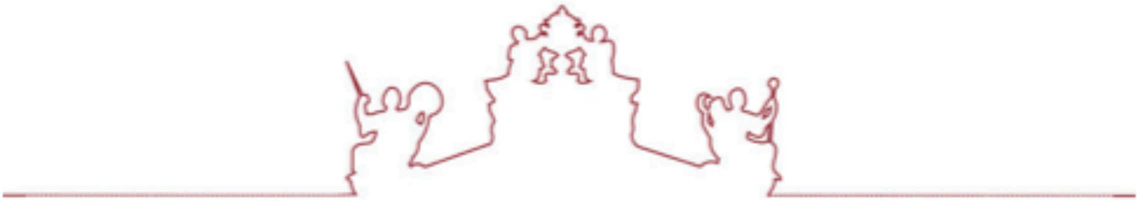

Universidade de Évora - Escola de Ciências e Tecnologia Universidade de Lisboa - Instituto Superior de Agronomia

Mestrado em Gestão e Conservação de Recursos Naturais

Dissertação

Valuing the acorn cupule of different oak species in a circular economy context

Ana Catarina da Torre Caeiro

Orientador(es) | Joana Amaral Paulo
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A dissertação foi objeto de apreciação e discussão pública pelo seguinte júri nomeado pelo Diretor da Escola de Ciências e Tecnologia:

Presidente	Pedro R. Almeida (Universidade de Évora)
Vogais	Jorge Gominho (Instituto Superior de Agronomia (ISA)) (Orientador) Luís Jorge Abreu Chorão de Quelhas Duarte () (Arguente)

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Resumo

Valorização das cúpulas de bolota de diferentes espécies de carvalho num contexto de economia circular

As cúpulas são consideradas um subproduto da fileira da bolota, a qual se encontra atualmente em franco desenvolvimento no nosso país. No entanto, estudos relacionados com a sua composição química são praticamente inexistentes. No presente trabalho, as cúpulas de três espécies autóctones de carvalho (Q*uercus suber, Quercus ilex*, Q*uercus pyrenaica*) foram analisadas quimicamente relativamente aos seguintes parâmetros: teores em cinzas, extrativos totais, lenhina total e açúcares; conteúdo em compostos fenólicos; atividade antioxidante. Os resultados demonstraram que todas as cúpulas são caracterizadas por altos níveis de extrativos totais e elevada capacidade antioxidante. *Q. pyrenaica* destacou-se pela quantidade de extrativos totais (34,1%). *Q. ilex* demonstrou maior percentagem de lenhina (32,9%) e açúcares (50,2%), flavonoides (450mgCat/gExt) e taninos condensados (114mgCat/gExt). *Q. suber* apresentou maior capacidade antioxidante (1894mgTrolox/gExt; IC50=3; AAI=8) e quantidade de fenóis totais (629mgCat/gExt). O conhecimento das composições químicas das cúpulas permite potenciar a sua futura utilização em novos produtos de base biológica.

Palavras-chave: Atividade antioxidante; Economia circular; *Quercus suber; Quercus pyrenaica; Quercus ilex*

English Summary

Acorn cupules are considered a by-product of the acorn sector, which is currently undergoing rapid development in our country. However, studies related to its chemical composition are practically non-existent. In the present work, cupules from three autochthonous species of oak (*Quercus suber, Quercus ilex*, *Quercus pyrenaica*) were chemically analysed for the following parameters: percentage of ash, total extractives, total lignin and sugar; content of phenolic compounds; antioxidant activity. The results demonstrated that all cupules are characterized by high levels of total extractives and great antioxidant capacity. *Q. pyrenaica* stood out for the amount of total extractives (34,1%). *Q. ilex* demonstrated a higher percentage of lignin (32,9%) and sugar (50,2%), flavonoids (450mgCat/gExt) and condensed tannins (114mgCat/gExt). *Q. suber* showed greater antioxidant capacity (1894mgTrolox/gExt; IC50=3; AAI=8) and content in total phenols (629mgCat/gExt). Knowledge regarding the chemical compositions of cupules allows us to enhance their future use in new bio-based products.

Keywords: Antioxidant activity; Circular economy; *Quercus suber; Quercus pyrenaica; Quercus ilex*

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1. Introduction

1.1 Oak trees (*Quercus*)

Oak trees belong to the largest genus in the Fagaceae family, *Quercus*. Together, they account for more than 600 species and are distributed worldwide, being important members of many forest communities, from temperate deciduous forests to subtropical and tropical savannahs (Tantray et al., 2017; Tejerina et al., 2011), where they serve as shelter and food source for a large amount of fauna of both vertebrates and invertebrates (Mason and Nesbitt, 2009; Turner et al., 2011). *Quercus* trees have been considered an evolutionary success for centuries due to their survival ability (Sork et al., 2022). This genus presents high variability between species (Castro-Vázquez et al., 2013; Nogueira et al., 2022; Akcan et al., 2017; Vinha et al., 2020) and is characterized by their wood, their simple, alternate leaves, and their fruits, the acorns (Tantray et al.,2017). In addition, they are also characterized by high variability between individuals of the same species, observed in a diversity of tree features and components (Kleinschmit, 1993; Paulo et al., 2011; Paulo et al., 2017).

Besides their ecological value, oaks also exhibit economic importance for many communities, such as in the region of Northern China (Wang X., 2013), the Mediterranean basin (Villar-Salvador et al., 2013) or the Eastern United States (Knoot et al., 2010), being exploited for many different purposes like construction materials, charcoal production, firewood, production of rope, cork, and the extraction of tannins for industrial use (Vinha et al., 2020; Guo et al., 2021; Lei, 2002). Oak wood is often used in coppering for grape wine aging due to its high content of ellagitannins and low molecular weight phenols (Castro-Vázquez et al., 2013; Zhang et al., 2015). In Morocco, *Q. ilex* is used to treat skin and throat infections and gastrointestinal disorders like diarrhoea and gastritis (Berahou et al., 2007). In Mexico, leaves of several *Quercus* species are consumed as food and used in traditional medicine as anticarcinogenic agents (Rocha-Guzmán et al., 2007). In Portugal, cork from *Q. suber* constitutes an important resource for the production of various sustainable and natural products like fabrics, shoes and bottle stoppers and this country is currently the world leader in the production of cork, delivering 55 % of the world's cork production (Camilo- Alves et al., 2020).

In Portugal, oak trees are abundant, occupying an area of around 1.107.600 ha, an area much higher than the one used to produce chestnuts (41.100 ha) or almonds (36.530 ha) (Cantos et al., 2003; Tejerina et al., 2011; INE, 2014). In the Portuguese territory, there are around 300 oak species, the most predominant being the Portuguese oak (*Q. faginea*), the Pyrenean oak (*Q. pyrenaica)*, the holm oak (*Q. ilex*), the cork oak (*Q. suber)* and the common oak (*Q. robur*) (Nogueira et al., 2022). The cork oak, *Q. suber*, is Portugal's second most abundant tree species, occupying around 720 thousand acres (ICNF, 2019). It is a central part of the local silvopastoral system called *montado*, which are very important for rural development, and it is famous for its cork production in the trunk and branches (Pereira & Tomé, 2004). The Holm oak, *Q. ilex*, occupies around 350 thousand acres in Portugal (ICNF, 2019) and is also frequently found in "*montados*". Lastly, the Pyrenean Oak, *Q. pyrenaica*, is more common in the North of Portugal, where it forms vast Pyrenean oak forests that range from Montesinho Natural Park until Serra da Nogueira. These forests are important habitats for biodiversity and have many agroecological and socioeconomic functions for the human communities around them, being essential for the sustainability of the silvopastoral systems in the region and providing various non-wood forest products like mushrooms, medicinal herbs and acorns. *Q. pyrenaicas'* wood is also very used in the region for the cooperage of wine.

1.2 Polyphenolic Compounds and Antioxidant Activity

Polyphenolic compounds are a diverse group of molecules with at least one phenol group in their chemical structure, meaning one or more hydroxyl groups on an aromatic ring (Manach et al., 2004). They are secondary metabolites produced by higher plants, involved in their defence, for example, against radiation, pathogens, or herbivory (Manach et al., 2004; Wall, 2010; Xiao et al., 2008). These compounds differ in the number of phenol rings and the elements that bind those rings to one another and include flavonoids, phenolic acid, and tannins (Rtibi et al., 2017; Manach et al., 2004).

The interest of polyphenolic compounds is that some have antioxidant properties, conferring them the ability to scavenge for oxygen free radicals and interrupt their chain reaction by forming resonance-stabilized phenoxyl radicals (Rocha-Guzmán et al., 2009; Soobrattee et al., 2005; Senol et al., 2018; Gezici et al., 2017; Orhan et al., 2012) and, thus, being potent natural biocompounds with anti-inflammatory, antimicrobial, hepatoprotective, gastro-protective, anti-oxidative and astringent activity (Andrensˇek et al., 2004; Moharram et al., 2015; Popović et al., 2013; Zdravkovic et al., 2015; Manach et al., 2004; Dillard and German, 2000).

Free radicals are formed normally during cell functions as intermediates of enzymatic reactions. Still, because they are very unstable, they can create oxidative damage to the cell constituents, which, in the long term, can cause diseases associated with oxidative stress, such as cancer, cardiovascular and neurodegenerative disorders, like Alzheimer's disease (Rocha-Guzmán et al., 2009; Ceriello and Motz, 2004; Uttara et al., 2009). It is important to find natural antioxidants because they exhibit less resistance by patients and do not show adverse effects, as is often the case with their synthetic counterparts (Cannas et al., 2015; Roleira et al., 2015; Nogueira et al., 2022; Jamuna et al., 2015).

1.3 Acorns

Acorns, the fruits of oak trees (Figure 1), have been an important resource for human societies throughout history (Khan et al., 2022). They have been a part of the human diet in various communities, from Native American Indians to modern European countries (Vinha et al., 2020; Wu et al., 2022), being consumed directly or in the form of flour to make bread or beverages like coffee or liqueur (Nogueira et al., 2022; Gezici & Sekeroglu, 2019; Vinha et al., 2020; Rakicá et al., 2005; Taib et al., 2020; Vacik et al., 2020). In China, acorns are used to make different foods like tofu, noodles, sauces, and cakes (Zhang & Wang, 2009; Zhang et al., 2014). Acorn oil is also used in some parts of the world for cooking or treating burns, skin irritation, and eczema (Taib et al., 2020). In times of war, acorns served as emergency food (Łuczaj et al., 2014). In Europe, the main application of acorns is as feed for livestock, especially for Iberian pigs and cattle (Taib et al., 2020). Additionally, in traditional medicine, acorns are used for many purposes, like treating haemorrhoids and kidney stones (Akcan et al., 2017), diabetes (Gezici & Sekeroglu, 2019), dysentery and other enterogastric (Tian, 2017) and inflammatory diseases (Sheu et al., 1997; Xin et al., 2008).

Due to the diversity of potential applications, a lot of interest has been developed in the chemical composition of this fruit (Rakicá et al., 2005; Akcan et al., 2017; Vinha et al., 2020; Ry et al., 2020). Acorn is recognized as having a high nutritional value, higher than cereals (Deforce et al., 2009; Rababah et al., 2008), with their main constituent being carbohydrates (75% to 84%), mainly starch (51 to 57%) (Silva et al., 2016). Other constituents including fibre (10-18%), fat (8-14%) and proteins (4-5%) (Silva et al., 2016). It is also known that acorns are a source of compounds with strong antioxidant activity, such as tannins, polyphenols and tocopherols, conferring them interesting biological activities like antimicrobial, antiulcerogenic, anti-inflammatory, antimutagenic, anticarcinogenic and anti-aging (Lee et al., 1992; Mamedova et al., 1993; Cantos et al., 2003; Rakić et al., 2006, 2007; Tejerina et al., 2011; Akcan et al., 2017; Söhretoglu et al., 2007; Shi et al., 2019; Taib et al., 2020; Łuczaj et al., 2014; Wang et al., 2020).

Several studies have focused on these biological functions and their applicability for the treatment of different human health conditions:

- Custódio et al. (2013) found acorns from *Q. suber* and *Q. ilex* to be a valuable source of biomolecules capable of alleviating symptoms associated with Alzheimer’s disease and other neurodegenerative disorders.

- Rtibi et al. (2017) studied the effects of acorns from *Q. ilex* on gastrointestinal physiological parameters *in vitro* and *in vivo*. They found that the aqueous extract from these acorns effectively reduces diarrhoea, fluid accumulation, electrolyte transport, and glucose absorption while exhibiting no toxicity.

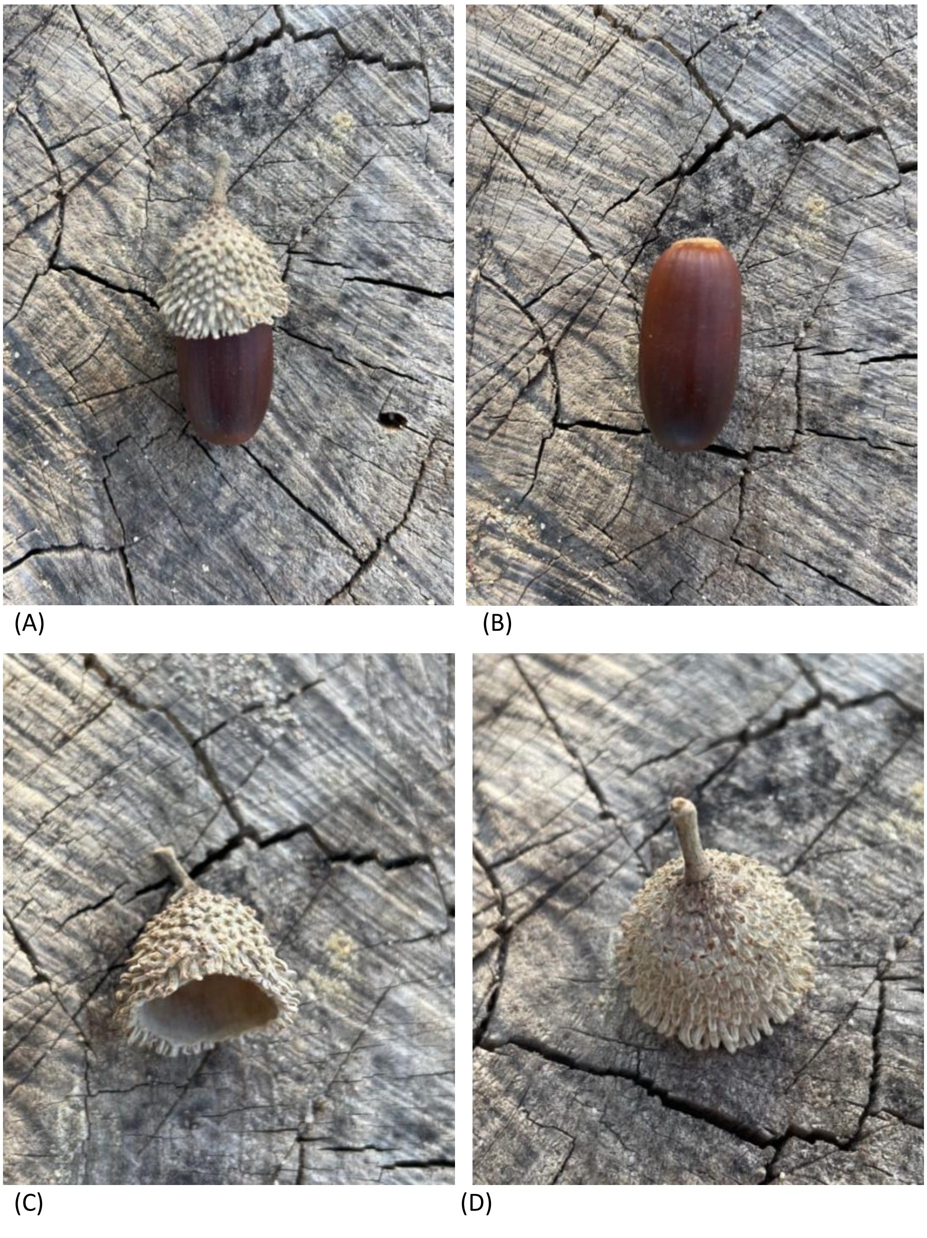

Figure 1.1 - Pictures from a Q. suber acorn, showing a complete acorn (A), an acorn without the cupule (B) and the acorn cupule (C and D).

- Lei et al. (2018) and Huang et al. (2016) isolated seventeen and ten triterpenoids from the Chinese acorns and the acorns of *Q. serrata*, respectively, and all the triterpenoids in both studies showed potent anti-neuroinflammatory activity.

- Nogueira et al. (2022) discovered the potential of acorns from *Q. robur* as anti-diabetic agents, with its extracts showing stronger inhibition of α-glucosidase compared to acarbose, the positive control.

Like this, the possible uses for acorns are augmenting, as now they are being viewed as natural agents for reducing risks or symptoms of cardiovascular diseases, diabetes, microbial infection, and inflammatory diseases, and new research has been focusing on incorporating them into the human diet as a sustainable and functional food alternative (Nogueira et al., 2022; Vinha et al., 2020; Rababah et al., 2008).

Besides the health benefits for humans, acorns are also being investigated for a handful of other possible industrial applications, such as for fermentation brewing, a sizing agent in the textile industry, a retarder and plugging agent in the petroleum industry, and production of modified starch, organic acid, green fuel, and green membrane materials (Lei et al., 2018). Provine et al. (1939) found that acorn oil can produce a new lubricant with many advantages compared to ordinary mineral lubricant oils, including increasing the resistance to sludging. Some research is even focused on acorns and cupules as a source of bio-reinforcement for plastics due to their low cost and very interesting thermal and mechanical properties (Jabri et al., 2022).

The official data regarding acorn production in Portugal are limited to *Q. suber* and *Q. ilex*, based on a model from 1994 and made considering data collected in Spain (ICNF, 2019. IFN6 – Anexo Técnico). The estimates presented reach 310 thousand tons of acorn from *Q. suber* and 127 thousand tons from *Q. ilex* produced per year in Portugal (ICNF, 2019). The statistics found in grey literature account for 400 thousand tons of acorns produced per year in Portugal, of which 84 thousand tons are used for feedstock (Coelho, 2015). All these data should be observed carefully, since they do not allow to account the variation of acorn harvest and counter-harvest years, the effect of annual climate , or the variability between the production of individual trees and regions. Furthermore, models developed for Portugal are limited to predicting acorn production in *Q. ilex* (Rodrigues, 2009). Currently, Rios (under way) is developing a model to estimate the production of acorns and cupules of *Q. pyrenaica.* This will be the first model to focus on this species, and to include a separate model for the cupule production estimation.

With the rising of the possible uses for acorns, there’s a need to also study and investigate potential uses for the by-products discarded as waste in this industry, like leaves, shells, and oak cups, as, in large quantities, they can be a source of environmental pollution (Yin et al., 2019). Besides, these by-products generated might represent potential sources of specific bioactive phytochemicals with different industrial applications (Ito et al., 2002; Bedgood et al., 2005; Kim et al.,2012; Brossa et al., 2009; Cantos et al., 2003; Karioti et al., 2010). Many studies have already been focusing mainly on leaves but also other parts of oak trees:

- Yang et al. (2015) found that the shell waste of *Q. acutissima* is a good material for bio-ethanol production.
- Custódio et al. (2015) showed that methanolic leaf extracts from *Q. suber* exhibit strong inhibitory activity against enzymes related to inflammatory diseases, like hyperglycemia and Alzheimer's disease.
- With similar results, Tahmouzi (2014), Moreno-Jimenez et al. (2015), Güllüce et al. (2004) and Sánchez-Burgos et al. (2013) discovered the same antioxidant, anti-inflammatory, anticarcinogenic and antimicrobial activities in the leaf extracts from many *Quercus* species: *Q. brantii, Q. sideroxyla, Q. durifolia, Q. eduardii, Q. ilex, Q. grisea, Q. laeta, Q. obtusata, Q. resinosa*.
- Rocha-Guzmán et al. (2009) further showed that water infusions of mature and fresh *Q. resinosa* leaves decreased the oxidative process and other damage to DNA in transformed human cells (HeLa cells).
- Andrenšek et al. (2004) and Unuofin & Lebelo (2021) demonstrated that the cortex stems and leaves of *Q. robur* exhibit antioxidant and antimicrobial activity, which can benefit the wine, food, and pharmaceutical industries.
- Genç et al. (2012) and Rakić et al. (2006) found gallic acid, lignans, catechin, and chlorogenic acid as the main components of stems and barks of *Q. coccifera*.

1.4 Acorn Cupules

Acorn cupules or cups refer to the bowl-like structures that enclose the acorn base (Figure 1.1). They are an essential trait for the classification of the Fagaceae family, formed by the gathering and healing of bracts (Vinha et al., 2016). It is thought that cupules have several essential functions, including chemical and physical protection of acorns, provision of nutrients and aid in seed dispersal (Vinha et al., 2016; Yang et al., 2023), but not much research has focused on this structure. Nevertheless, a few studies have found considerable phenolic compounds and high antioxidant activity in acorn cupules (Yin et al., 2019). Others even found higher levels of total phenols in cupules than in other tissues of different Q*uercus.* species:

- Gezici & Sekeroglu (2019) compared the total phenolic contents, in vitro antioxidant effects, neuroprotective potential and enzyme inhibitory activities of the ethanol and water extracts from the shell, cupule and shelled acorn of *Q. coccifera* and found that the extracts from the cupules not only contained higher total polyphenolic contents but also exerted greater enzyme inhibitory contents.

- X. Yang et al. (2023) found higher expression of the gene UGT84A13, involved in the formation of gallotannin, in cupules than in acorns.
- Q. Yang et al. (2023) measured the tannin content in different tissues of the Chinese cork oak (*Q. variabilis*). They discovered that hydrolysable tannins are the predominant phenols in this species and that cupules contain them in much higher contents. They also found the expression of UGT84A13 to be higher in cupules.

Analysing the chemical composition of acorn cupules is an important step that should go along with developing the acorn industry. Only if we know the chemical composition of the cupule and its variations among different oak species will we be able to value this resource in a circular economy context, by finding new and possibly very useful applications for this waste product. The chemical constituents of acorn cupules may have potential uses in several industries:

- Biofuels and energy generation: acorn cupules may be a viable source of biomass to produce bioenergy. Through processes like pyrolysis or gasification, cupules can be converted into biofuels or used to generate heat and electricity (Chao et al., 2017).
- Biomaterials creation: Cupules are also suitable for developing bio-based materials. Because of their fibrous properties can be processed into bioplastics (Jabri et al., 2022), which have potential applications in various industries, including packaging, construction, and textiles.
- Organic fertilizers and soil amendments.
- Production of dyes: another possible use is the production of natural dyes with the pigments found in acorn cupules (Machado et al., 2022). These constitute a sustainable and eco-friendly alternative to synthetic dyes in the textile and dyeing industries.
- Alimentary industry: depending on their antioxidant activity, they can be used as natural food preservatives (Karioti et al., 2010).
- Cosmetic industry: this is a big bet as a possible application for the acorn cupules. Because cupules contain various bioactive compounds with antioxidant, anti-aging, and anti-inflammatory properties, which can benefit skincare and cosmetic formulations, they can be used to make creams, serums, masks, or lotions. Additionally, the pigments in acorn cupules can provide a range of natural warm brown tones, which can be utilized in various cosmetic formulations, including lipsticks, eyeshadows, and blushes. The fibrous and abrasive nature of acorn cupules can make them suitable as natural exfoliating agents: finely ground cupules or cupule powder can be incorporated into facial scrubs, body polishes, or exfoliating masks to help remove dead skin cells and promote a smoother complexion.

- Pharmacological Industry: the compounds found in acorn cupules may be used to prevent or alleviate symptoms of certain inflammatory diseases or to develop important natural medical products, like in the study by Durai et al. (2021), which found that cupules can be converted into a low-cost and eco-friendly, carbon-based electrochemical sensor, for successful non-enzymatic and label-free detection of a marker found in high concentrations in inflammatory diseases, in biofluids like serum and plasma (1GP).

1.5 In this study

This thesis was developed within the scope of the ongoing projects WilfFood and AcornDew, which focus on valuing non-wood forest products from a circular economy perspective. The acorn cupules of three different oak species (*Q. suber, Q. rotundifolia, and Q. pyrenaica*) were chemically analysed and compared. The following components were considered: quantification of extractives in dichloromethane, water and ethanol; composition of lignin; amount of sugars present; content in total phenols, flavonoids, and condensed tannins; evaluation of antioxidant activity; quantification of fixed carbon.

The hypothesis raised is that, as a result of being a defensive organ, the acorn cupules present an accumulation of defensive metabolites, some of which may be used in industries like cosmetics or pharmaceuticals.

The final objective is to contribute to adding value to acorn cupules, which are currently considered a waste of the acorn industry, giving it new applications and encouraging a more sustainable and circular system. Through this, we'll meet the circular economy principles, minimizing waste generation and promoting resource efficiency, while contributing to a more sustainable acorn industry.

2. Materials and Methods

2.1 Acorn sampling and sample preparation

Acorns from *Q. suber, Q. ilex,* and *Q. pyrenaica* were collected in October and November 2022. Due to the species' current distribution in Portugal, the first two species were harvested in Herdade do Freixo do Meio, Montemor-o-Novo, and the last in Parque Natural Montesinho, Bragança.

After being collected in the field, the acorns were stored in plastic bags and transported to the laboratory. There, they were dried at air room temperature and the cupules were separated from the acorn.

Then, 100 grams of cupules from each species were reduced to powder in a mill (Retsch SM 2000 for under 2 mm and Thomas Wiley for under 1 mm) and the moisture was instrumentally determined for each species using Ohaus MB120.

All the assays were made in triplicate for each species and the final results are presented as a mean of the three repetitions. These repetitions were also used to calculate the standard deviation for each assay.

2.2 Summative Chemical Characterization

The summative chemical characterization was made in the 40-60 mesh fraction. It included the determination of the following: ashes content by incineration (TAPPI T15 os-58), total extractives by solvent extraction with dichloromethane, ethanol, and water for 16h each in a Soxhlet apparatus using 0,5 grams for each repetition of the previously milled samples (TAPPI T204 cm-07), total lignin as the sum of Klason (TAPPI T222 om-11) and soluble lignin measuring the absorbance of the hydrolysate in a spectrophotometer at 205 nm (TAPPI UM 205 om-93).

The lignin content was corrected to extractives and ashes content.

The composition of the monosaccharides, uronic, and acetic acids was determined in the hydrolysate attained from lignin analysis by High-Pressure Ion-Exchange Chromatography with a pulsed amperometric detector (HPIC-PAD) and a High-Pressure Ion-exclusion Chromatography with a UV/Visible detector (HIPCE-UV). The neutral monosaccharides and uronic acids were determined by separation in a Dionex ICS-3000 HPIC-PAD system using an Aminotrap plus Carbopac PA10 column (250 mm x 4 mm); using a gradient of NaOH + CH_3COONa as eluent (1 mL/min flow) at 25 °C. The acetic acid was determined in a Waters 600 HIPCE-UV system with a Biorad Aminex 87H column (300 x 7.8 mm) at 210 nm. The values attained were corrected to extractives content.

2.3 Phenolic Content and Antioxidant Activity

Initially, 0.5 grams of samples were extracted in an ethanol/water solution (50/50, v/v) for 1 h at 40 °C using an ultrasonic bath. Then, the samples were filtrated to remove the insoluble material, and the liquid (the extract) was stored at 4 ^{o}C. The solid residue was dried overnight at 50 ^{o}C and for 1 h at 100 ^{o}C and then weighed to determine extraction yield.

The phenolic content was adapted from Miranda et al. (2016) and determined as total phenol, flavonoids, and condensed tannin contents. First, the Folin–Ciocalteu method (Singleton & Rossi, 1965) was used to calculate the total phenol content, with gallic acid and catechin as a standard. Second, the flavonoid content was determined by an aluminium chloride colorimetric assay (Zhishen et al., 1999), and the standard used was catechin. Third, the condensed tannins content was determined by the vanillin-H_2SO_4 method (Sun et al., 1998) using catechin as a standard. For all the assays, a standard curve was made with increasing concentrations of the standard solution (starting at zero) by measuring their absorbances by UV spectrophotometry with wavelengths of 765 nm for the phenols, 510 nm for flavonoids, and 500 nm for tannins. Then, similar increasing concentrations were created with the extract solution, and their absorbances were measured. Finally, the concentration of the extract solutions was extrapolated from the standard curve where y is the absorbance (measured) and x is the concentration.

To determine the antioxidant properties of each sample, two methods were used: the FRAP method (ferric reducing antioxidant power) and the DPPH method (free radical scavenging activity). The first was adapted from Benzie and Strain (1996) and is very similar to the ones described above: Trolox was used as a standard to make a standard curve, and the concentration of the extract solution was calculated through its equation, where y is the absorbance (measured by UV spectrophotometry with a wavelength of 595nm), and x is the concentration. The DPPH method, also using Trolox as a standard, was adapted from Miranda et al. (2016), creating high, medium, and low concentrations of each extract solution. Their absorbances were measured at a wavelength of 515 nm to calculate the percentage of inhibition of 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) for each solution, creating a line with 'concentration' in the x-axis and '% of inhibition' in the y axis. The IC50 (extract concentration required for 50% DPPH inhibition) was calculated using the equation for that line, with y=50. The antioxidant activity index (AAI=final DPPH concentration/IC50) (Scherer & Godoy, 2009) was also calculated.

2.4 Fixed Carbon content

For the fixed carbon analyses 1,5 grams of sample (in triplicate for each species) was submitted to 950 ^{o}C for 7 minutes and weighed. After correction for ash content, the remaining weight is the fixed carbon. The result is expressed as a percentage.

3. Results

3.1 Summative Chemical Characterization

For the summative chemical analysis, the ash content was very similar between species, with average values of 2,7 % for *Q. suber* and *Q. ilex* and 3,0 % for *Q. pyrenaica*. The average values for the percentage of extractives, lignin and sugars for the three species are presented from Figure 3.2 to Figure 3.4. Cupules from *Q. pyrenaica* exhibited the highest average percentage of total extractives (34,1 %), with the most part being from the water extraction (26,0 %), followed closely by *Q. suber* (32,3 %). The three species had similar percentages of extractives in Dichloromethane. The species with the highest percentage of lignin was *Q. ilex*, with 32,9 % of total lignin and 31,8 % of Klason lignin. *Q. suber* and *Q. pyrenaica* showed lower average values (23,6 % and 20,2 % of total ligning, respectively). *Q. ilex* also exhibited the highest percentage of sugars, with half of their content being sugars (50,2 %) and hemicelluloses as the most representative group (33,5 %). In Figure 3.4, the sugars were grouped in Hemicelluloses. The detailed results can be found in Annex 1.

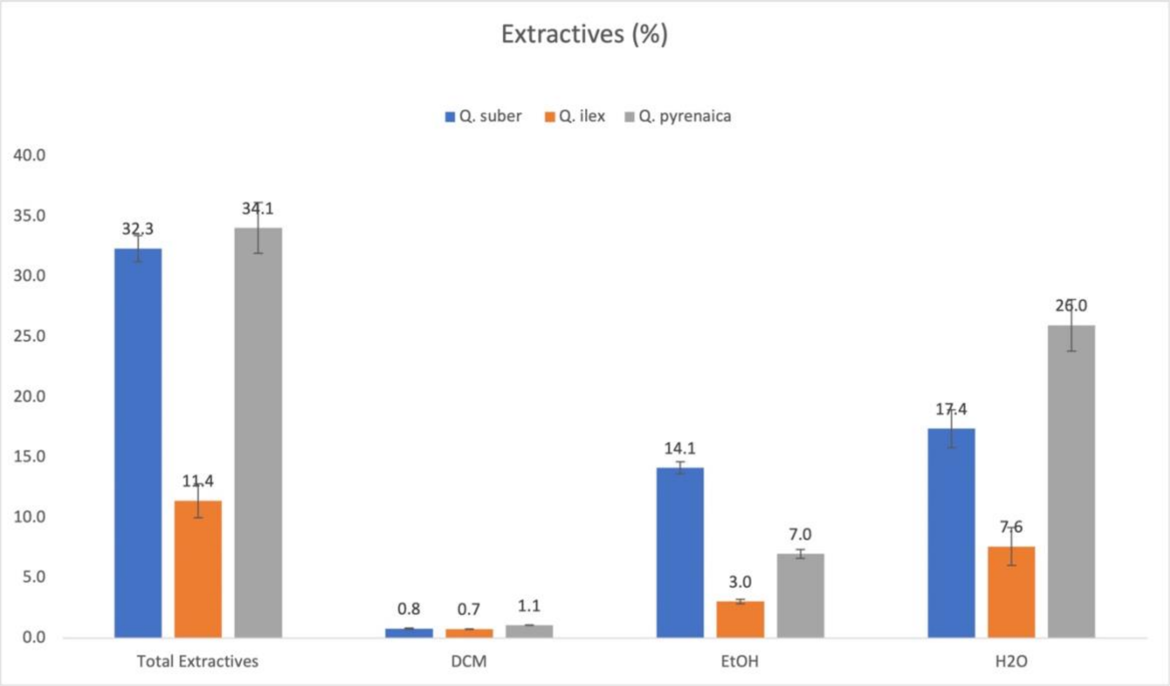

Figure 3.2 – Percentage of total extractives and extractives in Dichloromethane (DCM), Ethanol (EtOH) and Water (H20) after Soxhlet extraction of cupules from Q. suber, Q. ilex and Q. pyrenaica. The vertical line in each bar shows the standard deviation.

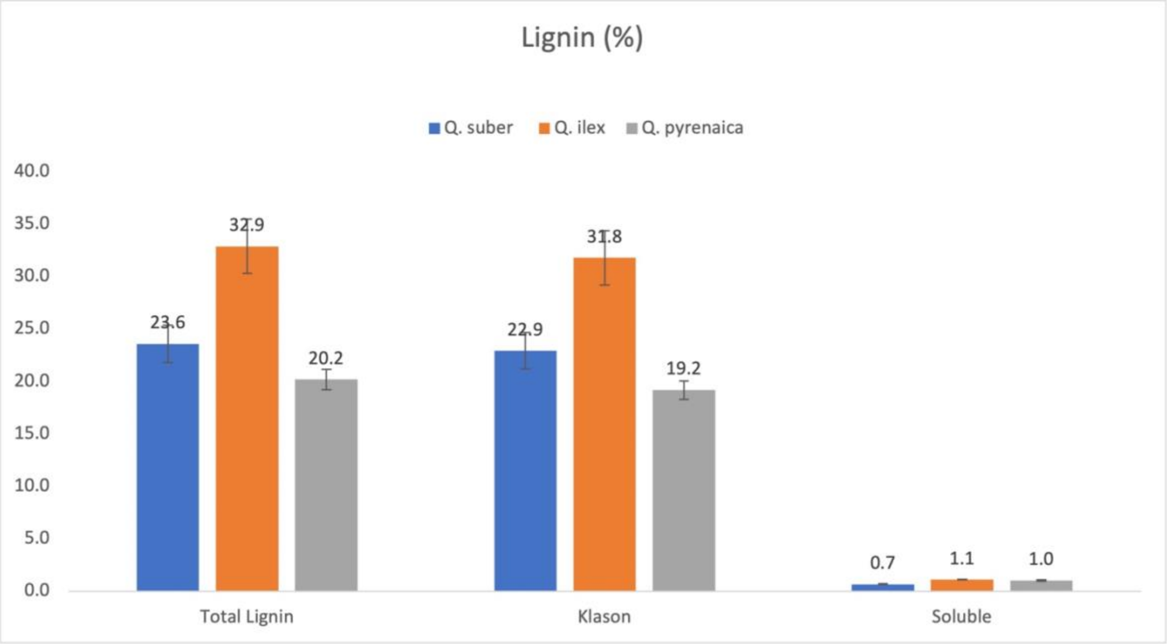

Figure 3.3 – The percentage of total lignin, Klason lignin and soluble lignin after Soxhlet extraction of cupules from Q. suber, Q. ilex and Q. pyrenaica. The vertical line in each bar shows the standard deviation.

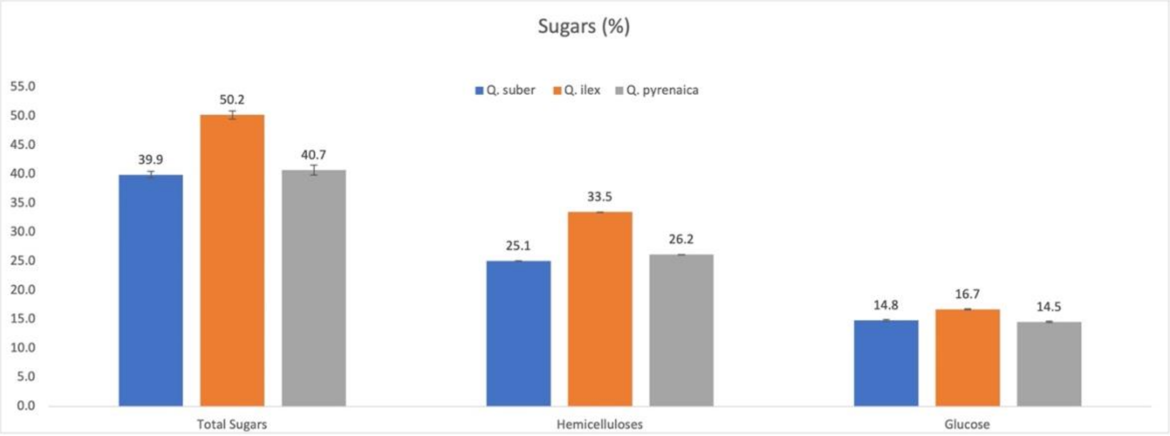

Figure 3.4 – Percentage of total sugars, Hemicelluloses and Glucose after Soxhlet extraction of cupules from Q. suber, Q. ilex and Q. pyrenaica Hemicelluloses include Ramnose, Arabinose, Galactose, Xylose, Mannose, Galacturonic acid, Glucuronic acid and Acetic acid. The vertical line in each bar shows the standard deviation.

3.2 Phenolic Content

The total phenols, flavonoids, and condensed tannins content was determined for each sample and is shown in Figure 3.5 to Figure 3.7. *Q. suber* had the highest content in total phenols, with 622 mgGAE/gExt or 629 mgCat/gExt. The content in flavonoids showed interesting results: Q. ilex had the highest content if expressed in milligrams of catechin equivalent per gram of extractive; however, when the units are converted to milligrams of catechin equivalent per gram of the original sample (milled solid), Q. suber had the highest content. Similarly, cupules from Q. ilex had the highest content in condensed tannins if in mgCat/gExt (114), but Q. suber exhibited the mgCat/gsample (25).

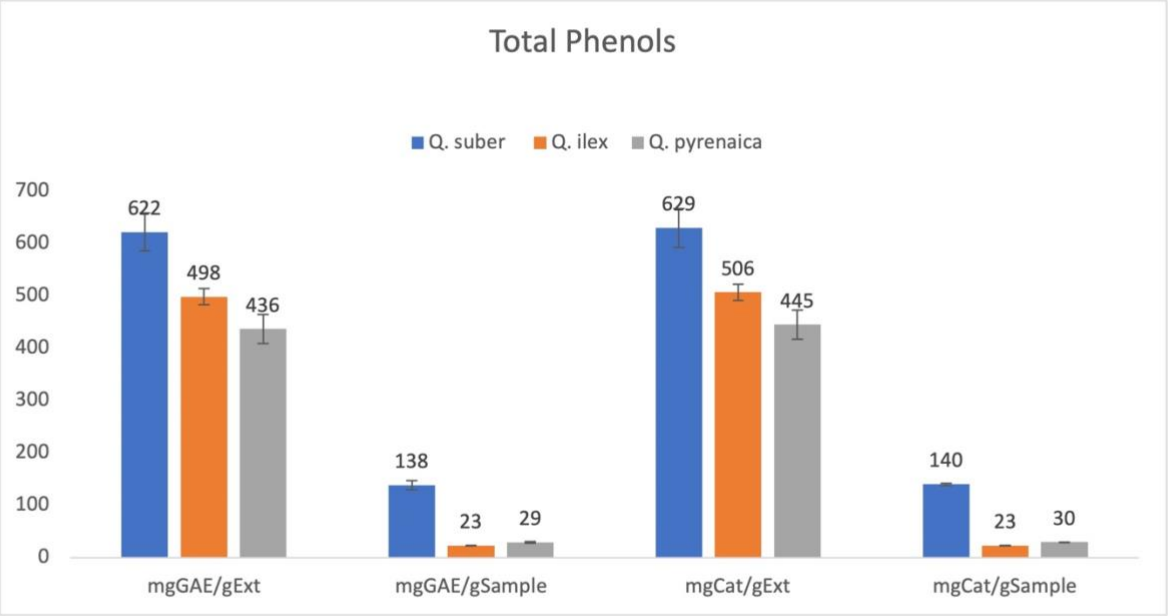

Figure 3.5 – Content in total phenols after the Folin–Ciocalteu method with gallic acid (GAE) and catechin (Cat) as a standard. mgGAE = milligrams of gallic acid equivalent per gram of extract (gExt) or gram of sample (gsample); mgCat = milligrams of cathequine equivalent. The vertical line in each bar shows the standard deviation.

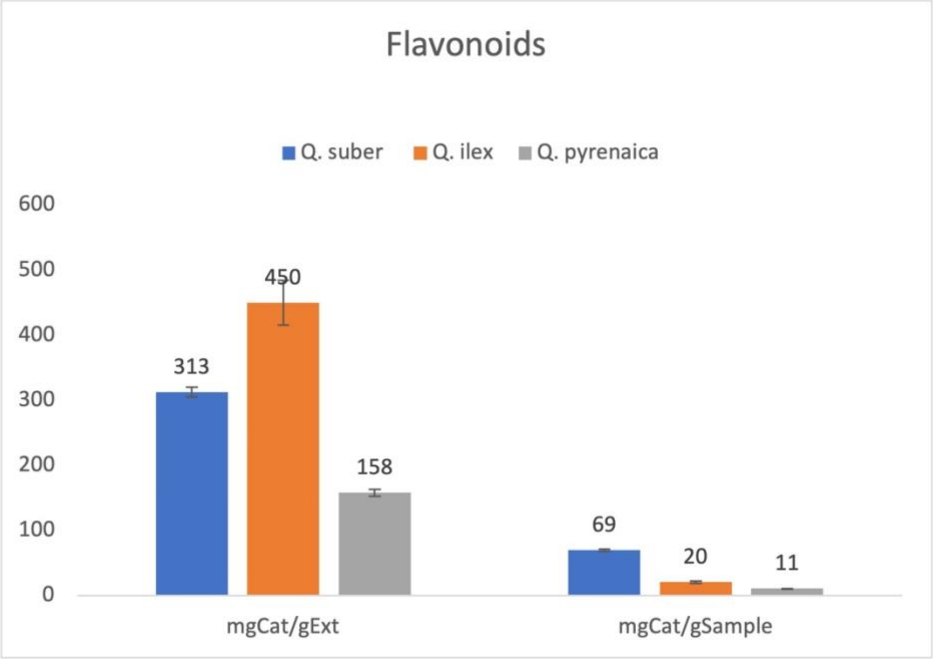

Figure 3.6 – Content in Flavonoids after an aluminum chloride colorimetric assay with cathequin as a standard. mgCat = milligrams of cathequine equivalent per gram of extract (gExt) or gram of sample (gsample). The vertical line in each bar shows the standard deviation.

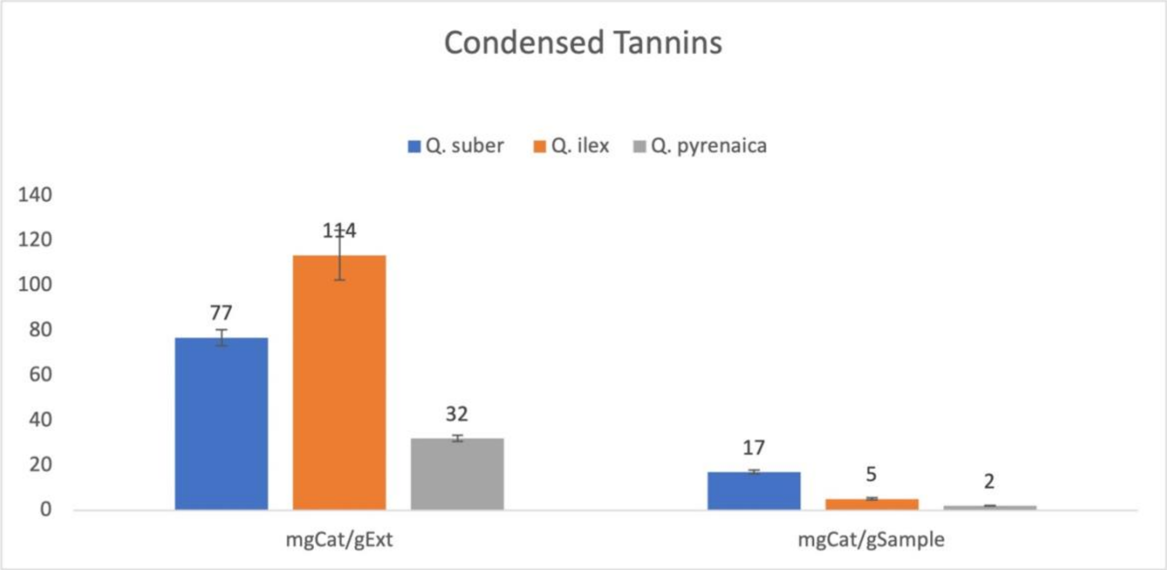

Figure 3.7 - Content in Condensed Tannins after the vanillin-H2SO4 method with cathequin as a standard. mgCat = milligrams of cathequine equivalent per gram of extract (gExt) or gram of sample (gsample). The vertical line in each bar shows the standard deviation.

3.3 Antioxidant Activity

All samples showed high antioxidant activity, which was observed in both methods (DPPH and FRAP). Cupules from *Q. suber* exhibited the highest antioxidant capacity, with an IC50 of 3 (3 µg of extract needed to inhibit 50 % of DPPH), an AAI (Antioxidant Activity Index) score of 8 (Figure 3.8) and a value of 1894 milligrams of Trolox equivalent per gram of extract (Figure 3.9). On the contrary, *Q. ilex* showed the lowest antioxidant capacity in both methods.

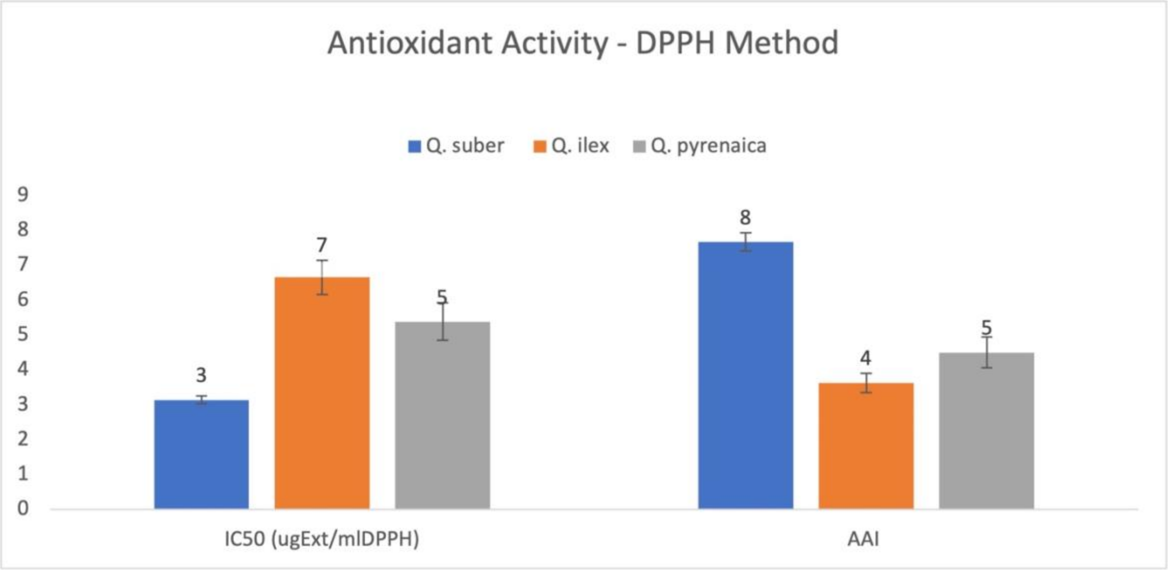

Figure 3.8 - Results of the DPPH method, used to determine the antioxidant activity of cupules from Q. suber, Q. ilex and Q. pyrenaica. IC50 measures the µg of extract necessary to inhibit 50 % of DPPH; AAI is the antioxidant activity index and

is calculated by dividing the IC50 obtained by the concentration of the solution of DPPH. The vertical line in each bar shows the standard deviation.

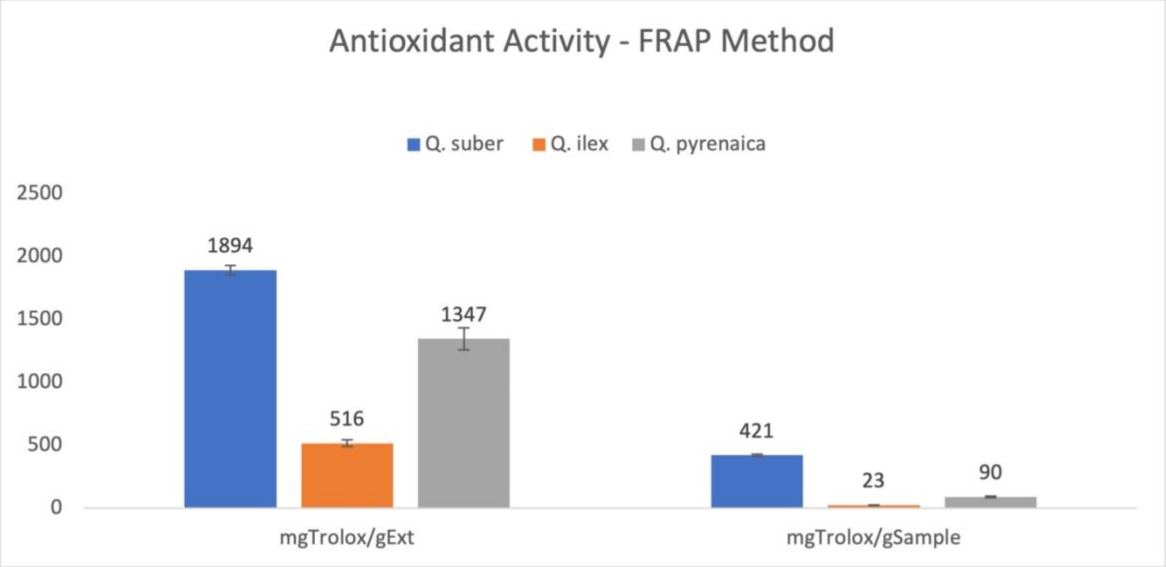

Figure 3.9 – Results of the FRAP method, used to determine the antioxidant activity of cupules from Q. suber, Q. ilex and Q. pyrenaica. Two units are shown: the mg of Trolox equivalent – per mg of extract (gExt) or per gram of sample (gsample). The vertical line in each bar shows the standard deviation.

3.4 Fixed Carbon content

Finally, fixed carbon analysis (Figure 3.10) revealed that all samples have similar percentages, with cupules from *Q. suber* with the highest (25,5 %), followed by *Q. ilex* (23,0 %) and *Q. pyrenaica* (19,9 %).

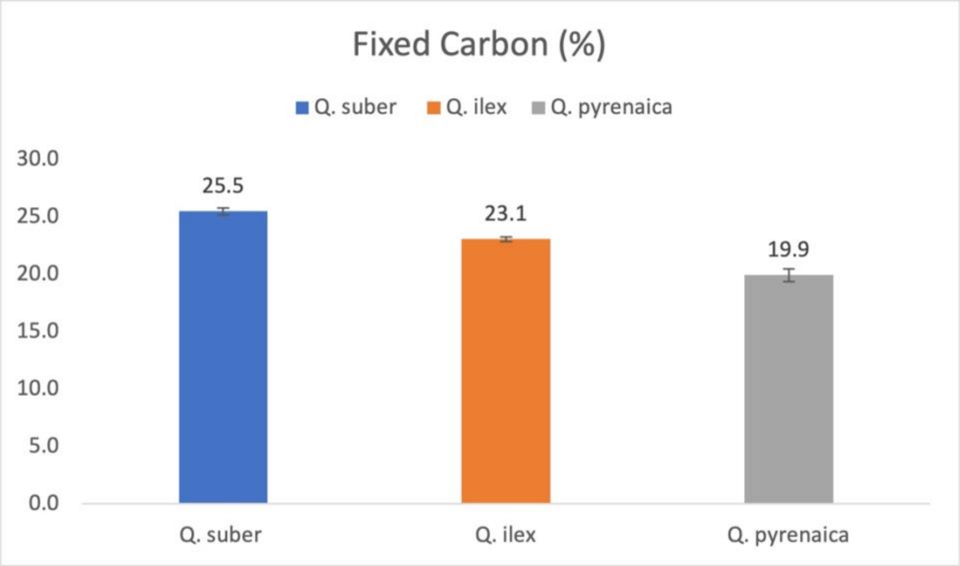

Figure 3.10 - Fixed Carbon content of cupules from Q. suber, Q. ilex and Q. pyrenaica. The vertical line in each bar shows the standard deviation.

4. Discussion

In this thesis, acorn cupules from three important *Quercus* Species in Portugal (*Q. suber, Q. ilex and Q. pyrenaica*) were chemically analysed concerning their ash content, total extractives, total lignin and percentage of sugars present. Their phenolic content was also determined by using three different assays to quantify total phenols, flavonoids and condensed tannins present in the extract with a 50,0 % ethanol/water solvent. Lastly, the antioxidant activity of that same extract was evaluated for the three species, making use of the FRAP and DPPH methods. All cupules exhibited high levels of total extractives, high phenolic content and very interesting antioxidant activities.

Q. suber and *Q. pyrenaica* showed similar and high percentages of total extractives. The highest percentage of extractives was obtained in water and ethanol for all species, meaning that most are polar. With dichloromethane, the percentages of extractives obtained are small and very similar for the three species. This indicates that these might not be relevant for the phenolic content and antioxidant activity tests performed afterward. On the other hand, the three species exhibited differences in extractives obtained with ethanol and water: for *Q. pyrenaica,* this difference is very noticeable, with more than triple extractives obtained with water; for *Q. suber*, the discrepancy is not that high; *Q. ilex* is in the middle, with more than double of extractives obtained with water. In this study, the compounds present in each extract were not analysed. However, these results show that some of the extracts in cupules from the three oak species tested differ. Further research should focus on identifying the individual compounds present after extraction with ethanol and water to understand their properties better. For example, it was found that the anticarcinogenic properties of green tea are mainly attributed to the antioxidant activities of epicatechins and epigallocatechin gallates, quercetin, kaempferol and myricetin (Moreno-Jimenez et al., 2015).

Additionally, the choice of extraction solvent must be based upon the objective of each study. Methanol is usually a better solvent for more consistent extraction of antimicrobial substances from medical plants (Güllüce et al., 2004). Berahou et al. (2007) further found ethyl acetate, butanol and aqueous extracts of *Q. ilex* bark to be effective in vitro against several bacterial strains, while hexane and dichloromethane extracts were almost inactive. Hayouni et al. (2007) concluded that high-polarity solvents extract compounds with significant effects on antioxidant activity, which is consistent with the results obtained in this study.

Q. ilex showed the lowest percentage of total extractives, but its content in lignin was the highest. With almost 33,0 % of total lignin present, *Q. ilex* cupules show higher percentages of lignin than most lignocellulosic materials found in the literature. They can be a source of this material for industrial uses such as the production of fuels or for obtaining low molecular weight chemicals

(Zevallos Torres et al., 2020). *Q. suber* and *Q. ilex* showed similar but lower percentages of total lignin. However, they are not behind compared to the literature and can also be considered for the same industrial uses as *Q. ilex*. Accordingly, the content in hemicelluloses for the cupules from the three species is high when compared to the literature (Zevallos Torres et al., 2020). Again, *Q. ilex* showed higher percentages than the other two species tested but the difference between the three is not as high. As polysaccharides that bind cellulose microfibrils together in the cell wall, hemicelluloses can have different applications, like producing biofuel and bioproducts such as packaging materials (Huang et al., 2021). Further research should focus on different extraction methods and their optimization to understand if cupules can be a viable source of lignin and hemicellulose.

There was a significant discrepancy between the extraction yield in both methods used in this study. The ultrasound extraction with 50,0 % ethanol/water solvent resulted in very small percentages of total extractives compared to the Soxhlet extraction with dichloromethane, ethanol and water (see Annex 2). The highest difference was found for *Q. pyrenaica,* with 34,1 % of total extractives in the Soxhlet method and 6,7 % in the ultrasound method. Since most of the extractives (especially for *Q. pyrenaica*) were found in water, it is possible that either the solvent used in the ultrasound method is not efficient or the extraction method should be different. Yin et al. (2019) found that the highest amount of total phenols was obtained when using a 50.0 % aqueous ethanol solvent. However, their extraction method was not the same as used in this study. Thus, if these cupules are to be used in industry, an efficient method and solvent must be researched to reach an optimized chain industrial reaction.

Regarding the phenolic content, the results are presented in two different units: milligrams of Gallic acid equivalent (GAE) or Catequin equivalent (CAT) per gram of extract (the extracted solution after ultrasound extraction with 50 % ethanol/water) or per gram of sample (the solid after milling). *Q. suber* had the highest content in total phenols using both units but only the highest content in flavonoids and condensed tannins if expressed in mgCat/gsample, indicating that the yield of ultrasound extraction for this species is higher. With less grams of sample necessary for the same amount of flavonoids or condensed tannins, cupules from *Q. suber* should be considered first for industrial purposes, especially if the extraction method is by ultrasound.

Depending on their structure, tannins can be divided into condensed or hydrolysable (Q. Yang et al., 2023). They are compounds with important properties, like antibacterial, astringent, antihemorrhagic and antidiarrhoeic, making them suitable for phytotherapy (Łuczaj et al., 2014). However, high levels of tannins can also be toxic, disturbing the absorption of nutrients (Chung et al. 1998), especially condensed tannins (Q. Yang et al., 2023). In this analysis, the content in condensed tannins was assessed. The results are similar to a study that focused on cupules from *Q.*

mongolica that found a value of 11,71 mgCAT/gsample of condensed tannins (Yin et al., 2019): only *Q. suber* had a higher value than this, while the other two species tested showed lower results. However, it has been demonstrated that hydrolysable tannins are the primary group of tannins found in oak cups (Onem et al., 2014; Yin et al., 2019). Thus, in the future, it is important to also analyse their content for the three species before making conclusions regarding using them for human consumption.

The structure of phenolic compounds rather than their quantity confers cupules' antioxidant capacity, as this is usually dependent on the numbers and positions of the hydroxyl groups in relation to the carboxyl functional group (Hayes et al., 2011). Yin et al. (2019) associated the content of flavonoids and condensed tannins, rather than the total phenolic content, with increasing antioxidant activity. Moreno-Jimenez et al. (2015) associated higher antioxidant activities with higher levels of gallic acid and catechins. In this study, two methods were used to investigate the antioxidant capacity of acorn cupules: the FRAP method, in which the ferric-reducing ability of the extract is evaluated, and the DPPH method, in which the power of the extract to inhibit DPPH is tested. Cupules from the three species tested showed very good antioxidant capacity, with *Q. suber* showing the best activity in both methods, exhibiting an IC50 of 3, meaning that only 3 µg of extract are needed to inhibit 50,0 % of DPPH. As DPPH is not a naturally occurring radical and is relatively stable compared to the highly reactive species responsible for oxidative damage in biological systems, these results show that the free-radical scavenging capacity of our samples, especially cupules from *Q. suber,* is associated with phenolic hydroxyl substituents with high reactivity.

On the contrary to the literature, in this study, the species with the highest content in flavonoids and tannins (mgCat/gExt) was *Q. ilex*, the species with the lowest results in both antioxidant methods. Like this, there are probably other phenolic compounds conferring *Q. subers’* and *Q. pyrenaicas’* cupules their higher antioxidant capacity. Furthermore, the compounds in the plants that confer their antioxidant activity are complex and diverse and may act through distinct mechanisms against oxidizing agents. Thus, to fully reflect the comprehensive antioxidant capacity of all the compounds present in the extracted solution, there’s a need to use various methods with different mechanisms.

This study is an initial step in finding applications for acorn cupules, a by-product of the acorn industry. Besides not having much research focused on acorn cupules, another difficulty was the incapacity to compare the results with other studies, due to the use of different extraction methods, solvents and analysis. Nevertheless, to our knowledge, this is the first study to focus on the cupules of these three oak species, which represent the Portuguese forest.

The results obtained are interesting and promising and further research should focus on investigating different geographic zones and seasons. It has been found that the content of phenolics, tannins and flavonoids can be altered between regions (Łuczaj et al., 2014; Castro-Vázquez et al., 2013) but also due to time of the year, exposure to light, and even factors like soil type and rainfall (Moreno-Jimenez et al., 2015; Manach et al., 2004). Łuczaj et al. (2014) further found that even the size and weight of acorns can influence the amount of polyphenols present, which could also be the case for cupules.

The extraction yield and its optimization are important steps before considering the application of cupules in any industry, as it is important to limit losses of bioactive compounds during industrial processing. Conventional separation methods of bioactive compounds include ion exchange, solvent extraction, high-speed counter-current chromatography, preparative high-performance liquid chromatography and solid-phase extraction and they all exhibit certain levels of organic solvent wastage and constitute a high cost and time-consuming procedure with low recoveries and environmental pollution (Li et al., 2015). Yin et al. (2019) used a one-step HPD-100 macroporous resin chromatography procedure as a simple and effective method of isolating bioactive compounds from acorn cupules. Onem et al. (2014) investigated optimizing the extraction parameters for solvent extraction of valonia tannin from the acorn cupules and beards with Box–Behnken experimental design. They found that each extraction parameter in the experimental design did not reveal significant differences in tannin content but significantly affected the extraction yield.

Moreover, public acceptance is an important topic to consider if cupules were to be utilized in cosmetics or human consumption. Rocha-Guzmán et al. (2012) showed that *Q.* leaves infusions had a higher content of gallic acid and catechins and showed the best antioxidant capacity in comparison to the two commercial green teas. However, the consumer preference for the first was much lower than for the second.

Lastly, other parameters to consider include the economic viability, safety and environmental impact of utilizing cupules.

5. Conclusion

This thesis investigated the chemical composition, the polyphenolic content and the antioxidant activity of acorn cupules from *Q. suber, Q. ilex* and *Q. pyrenaica*. The results showed differences between the three species in all the parameters studied, with *Q. ilex* being very different in the chemical summative characterization and *Q. suber* standing out in the antioxidant capacity. In the Soxhlet extraction, the three species showed low percentages of extractives with dichloromethane and all exhibited higher percentages of extractives with water. *Q. ilex* had the lowest percentage of total extractives, while *Q. suber* and *Q. pyrenaica* had similar percentages. However, *Q. pyrenaica* showed a significant discrepancy in the percentages obtained with water and ethanol, while *Q. suber* did not. Klason lignin was by far the most abundant in cupules from all species, with very similar and low percentages of soluble lignin. *Q. ilex* had the highest percentage of total and Klason lignin; again, *Q. suber* and *Q. pyrenaica* did not differ much, which was also true for the amount of sugars present. In this case, all species showed higher amounts of hemicelluloses than glucose and *Q. ilex* had the highest percentage of both types of sugar. *Q. suber* had the highest content in total phenols and the highest yield of ultrasound extraction. In the extracted solution, *Q. ilex* exhibited the highest content of flavonoids and condensed tannins, followed by *Q. suber*. The antioxidant capacity was high for all species, but *Q. suber* had the best results in both tests, followed by *Q. pyrenaica.* With these results, acorn cupules offer exciting possibilities as a valuable, abundant and low-cost natural source of antioxidants, lignin and/or hemicelluloses, with variable uses in many industries. Further research and development are required to fully exploit the benefits of acorn cupules, including studying their bioactive compounds in detail, optimizing the extraction process and processing techniques and evaluating their suitability for different industries.

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Annexes

Annex 1 - Summative chemical analysis for the cupules of Q. suber, Q. ilex, and Q. pyrenaica

	Q. suber	***Q. ilex***	***Q. pyrenaica***
Ashes (%)	**2,7 (± 0,12)**	**2,7 (± 0,06)**	**3,0 (± 0,12)**
Total Extractives (%)	**32,3 (± 1,08)**	**11,4 (± 1,40)**	**34,1 (± 2,10)**
DCM	0,8 (± 0,04)	0,7 (± 0,01)	1,1 (0,02)
EtOH	14,1 (± 0,49)	3,0 (± 0,20)	7,0 (0,38)
H2O	17,4 (± 1,57)	7,6 (± 1,58)	26,0 (± 2,14)
Total Lignin (%)	**23,6 (± 1,76)**	**32,9 (± 2,59)**	**20,2 (± 0,96)**
Klason	22,9 (± 1,73)	31,8 (± 2,57)	19,2 (± 0,88)
Soluble	0,7 (± 0,03)	1,1 (± 0,02)	1,0 (± 0,08)
Sugars (%)	**39,9 (± 0,59)**	**50,2 (± 0,70)**	**40,7 (± 0,85)**
Ramnose	0,3 (± 0,01)	0,5 (± 0,01)	0,3 (± 0,01)
Arabinose	1,7 (± 0,15)	2,4 (± 0,08)	0,8 (± 0,12)
Galactose	1,5 (± 0,05)	1,8 (± 0,03)	0,9 (± 0,04)
Glucose	14,8 (± 0,27)	16,7 (± 0,24)	14,5 (± 0,39)
Xylose	16,7 (± 0,30)	22,0 (± 0,16)	19,2 (± 0,49)
Mannose	-	-	-
Galacturonic acid	1,1 (± 0,04)	1,3 (± 0,08)	-
Glucuronic acid	0,1 (± 0,08)	0,2 (± 0,003)	-
Acetic acid	3,7 (± 0,16)	5,3 (± 0,19)	4,9 (± 0,47)

Annex 2 - Phenolic content (total phenols, flavonoids, and condensed tannins) for cupules of Q. suber, Q. ilex, and Q. pyrenaica. (GAE=Gallic acid equivalent; Ext=Extract; CAT=Cathequin equivalent.).

	Q. suber	***Q. ilex***	***Q. pyrenaica***
% of extractives from Ultrassound Extractives	**22,2** (± 0,29)	**4,5** (± 0,34)	**6,7** (± 0,42)
Total phenols (mgGAE/gExt)	**622** (± 36,32)	**498** (± 15,55)	**436** (± 27,39)
Total phenols (mgGAE/gsample)	140 (± 8,29)	23 (± 0,71)	30 (± 1,86)
Total phenols (mgCat/gExt)	**629** (± 37,28)	**506** (± 15,71)	**445 (± 27,92)**
Total phenols (mgCat/gsample)	140 (± 1,84)	23 (± 0,03)	30 (± 0,12)
Flavonoids (mgCat/gExt)	**313** (± 7,90)	**450** (± 34,58)	**158** (± 4,95)
Flavonoids (mgCat/gsample)	69 (± 1,76)	20 (± 1,56)	11 (± 0,33)
Condensed Tannins (mgCat/gExt)	**77** (± 3,64)	**114** (± 11,05)	**32** (± 1,38)
Condensed Tannins (mgCat/gsample)	17 (± 0,81)	5 (± 0,50)	2 (± 0,09)

Annex 3 - Values for the two methods used (FRAP and DPPH) for the determination of antioxidant activity in cupules of Q. suber, Q. ilex, and Q. pyrenaica.

	Q. suber	***Q. ilex***	***Q. pyrenaica***
FRAP (mgTrolox/gExt)	1894 (± 38)	516 (± 27)	1347 (± 87)
FRAP (mgTrolox/gsample)	421 (± 8,46)	23 (± 1,21)	90 (± 5,76)
IC50 (ugExt/mlDPPH)	3 (± 0,11)	7 (± 0,49)	5 (± 0,54)
AAI	8 (± 0,26)	4 (± 0,27)	5 (± 0,44)