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Exploring acorn shells: Phenolic composition and bioactive potential for sustainable valorization

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ABSTRACT

Pedunculate (*Quercus robur* L.), holm (*Quercus rotundifolia* Lam.), and cork (*Quercus suber* L.) oaks are abundant across the Portuguese landscape. This study aims to evaluate the phenolic composition and bioactivities of acorn shell samples and determine their potential as a functional compound source. In total, five acorn shell samples collected in different locations and from different species were analyzed: *Q. rotundifolia* (*Q. rot-1* and *Q. rot-2*), *Q. suber* (*Q. sub-1* and *Q. sub-2*) and *Q. robur* (*Q. rob-1*). A total of nine phenolic compounds were tentatively identified, namely gallic and ellagic acids and derivatives. Digalloyl hexoside was the compound detected in higher concentrations in all extracts (2.093 – 8.3 mg/g extract). *Q. suber* samples exhibited the lowest IC_{50} values for TBARS assay, lower than the positive control used (Trolox). Overall, the studied samples demonstrated the capacity to inhibit the proliferation of all tumor cell lines tested. Sample *Q. sub-1* demonstrated the most promising antibacterial capacity. According to the results, the acorn shell extracts exhibited promising potential, and it may be interesting to conduct a deeper study on the samples of this species.

1. Introduction

The genus *Quercus*, a member of the Fagaceae family, includes important woody plants with simple alternating leaves, wind-pollinated blooms, and exceptional lifespan. *Quercus* trees have been an incredible resource for civilization since prehistoric times. The products derived from these trees are utilized to produce tannins for tanning leather, as well as wood for construction and energy production through combustion. With approximately 600 species globally, the genus *Quercus* is a significant clade of woody angiosperms, notable for its species diversity, ecological dominance, and economic value, particularly in the northern hemisphere. Oaks prosper in diverse habitats, ranging from temperate deciduous forests to subtropical and tropical savannahs, and are adaptable to various well-drained soils. Their prevalence underscores their importance in ecosystems worldwide (Gea-Izquierdo et al., 2006; Taib et al., 2020; Tantray et al., 2017).

Quercus species, especially pedunculate (*Quercus robur* L.), holm (*Quercus rotundifolia* Lam.), and cork oaks (*Quercus suber* L.) are abundant across the Portuguese landscape, with an estimated total of 1.2 million hectares, and incorporating approximately 36 % of the country's total forested area (Ezequiel, 2021). Of those 36 % of Portuguese forest occupation, 94.44 % corresponds to cork and holm oak and 5.56 % to the remaining oak species (2 % of the total continental Portuguese forest territory).

Acorns are small nut-like fruits produced by different species from the *Quercus sp.* genus. These fruits play a crucial role in the ecosystems. Acorns serve as a vital food source for animals, including squirrels, deer, birds, and insects, and are also a nutrient-rich source; namely of starch, protein, fat, and essential minerals like calcium, phosphorus, potassium, and magnesium (Sekeroglu et al., 2017). The high concentration of compounds of interest present in the different *Quercus* species and the availability of acorns (free or affordable) make this fruit a sustainable candidate for the application of acorn extracts in different industries (Górnaś., 2019a). Acorns are rich in phenolic compounds (Martins et al.,

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2022; Oracz et al., 2022) such tannins, and, in addition to the traditional use of these compounds in the leather industry for dyeing and skin treatments, tannins could also be used in adhesives, insulating foams, winemaking, animal nutrition, mineral and petroleum industries, water treatment and metallic protection (Das et al., 2020). Regarding lipophilic compounds, acorns contain beneficial unsaturated fatty acids, particularly oleic acid, along with vitamin E (Akcan et al., 2017). Górnaś et al., (2019b) described that *Q. rubra* and *Q. robur* oak species exhibit a higher abundance in tocopherols, namely β- and γ- isoforms, and, therefore, can be exploited as a source of these compounds for sectors such as chemical, cosmetic, and pharmaceutical. In addition, in a study carried out by Şahin and Saka (2013), acorn shells were used to produce activated carbons using CO_2 and H_2O activation with a $ZnCl_2$–HCl pretreatment at 850 °C. The authors observed that the obtained activated carbons exhibit the potential to be used for adsorption applications depending on the various activation settings.

Their rich chemical composition is essential for the diverse properties that have been associated with this species. Relegated for animal feed for centuries and exploited as a fallback food in famine times, this fruit is now gaining recognition as an emerging alternative and competitive food source. Acorn flour also shows potential for enhancing the rheological properties of bakery products and as a source of functional compounds, normally absent in commonly used flours (Vinha et al., 2016).

Despite the growing interest in exploiting the acorn for different uses, the shell of this fruit continues to be an underexplored resource; therefore, this study aims to analyze its phenolic composition and several bioactive properties and verify if it exhibits potential to be exploited as a source of new bio-based products.

2. Materials and methods

2.1. Sample preparation and extracts obtention

The *rotundfolia*, *suber*, and *robur* species of acorn samples were studied. The samples from *Quercus rotundifolia* Lam. species were collected in Bragança and Portalegre (*Q. rot-1* and *Q. rot-2*, respectively), while *Q. suber* L. (*Q. sub-1* and *Q. sub-2*) were collected in Braga (Portugal). Finally, a sample of *Quercus robur* L. collected in Braga was also studied (Table 1). The studied acorn samples went through a manual peeling process to separate the shell from the acorn body. All samples come from batches of at least 100 kg of mature acorns, i.e., fallen on the ground and with > 90 % fully ripened fruits, collected and processed by the Landratech company (Azambuja, Portugal) between September and November. The batches were dried in an oven with air extraction at a nominal temperature of 50 ºC, with a real chamber temperature ranging between 35 and 55 ºC. Finally, after drying, the shells were ground using a disc mill (Oukaning, China).

The shell samples studied were subjected to an aqueous extraction with a solid/liquid ratio of 25 g/L for 1 hour at 40 °C with constant stirring; the extraction process was performed twice. After filtration, the samples were freeze-dried (FreeZone 4.5, Labconco, Kansas City, MO, USA). The acorn shell extracts obtained were stored under protection from the light and froze at −20 ºC until further analysis.

2.2. Phenolic compounds analysis

The determination of the acorn shell extracts phenolic compounds was conducted through high-performance liquid chromatography coupled to a mass spectrometry (HPLC-DAD-ESI/MSn). A Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system equipped with a diode array detector coupled to an electrospray ionization mass detector, a quaternary pump, an auto-sampler (kept at 5 °C), a degasser and an automated thermostatted column compartment was used. To prepare the samples, the extracts were redissolved in water at a final concentration of 10 mg/mL and subsequently filtered through an LC filter disk (nylon filter, 0.22 μm pore size, 25 mm diameter, Whatman M, GE Healthcare, Buckinghamshire, UK). Double online detection was performed using a Diode Array Detector (DAD) at preferred wavelengths of 280 nm and 370 nm. Additionally, a mass spectrometer (MS) was connected to the HPLC system through the DAD cell outlet for enhanced detection, following a previously established procedure (Bessada et al., 2016). For quantitative analysis, calibration curves derived from commercial standards were utilized, and the obtained results were expressed in milligrams per gram of extract.

2.3. Antioxidant activity

The antioxidant activity of acorn samples was analyzed through two *in vitro* assays, the thiobarbituric acid reactive substances (TBARS) formation and the cellular antioxidant activity (CAA).

For the TBARS assay, serial dilutions of the obtained extract redissolved in water were prepared, ranging in concentration from 625 μg/mL to 0.61 μg/mL. The assessment of extracts' lipid peroxidation inhibition was performed by the incubation of the different concentrations tested with the porcine (*Sus scrofa*) brain and following the procedure previously described by Pinela et al. (2012). The color intensity of the malondialdehyde–thiobarbituric acid (MDA–TBA) complex was quantified by determining its absorbance at 532 nm. The results were expressed as the concentration of the extract responsible for inhibiting lipid peroxidation by 50 % (EC_{50}).

For the CAA assay, the extracts were re-dissolved in water to obtain a concentration of 8 mg/mL, from which successive dilutions were performed with 2′,7′-dichlorohydrofluorescein (DCFH, 50 mM) prepared with ethanol and diluted with Hank's Balanced Salt Solution (HBSS), obtaining the final concentrations tested (500–2000 μg/mL). The different concentrations of extracts were incubated with previously prepared murine macrophage cells (RAW 246.7). This procedure was followed as described by de la Fuente et al., (2022). The obtained results were expressed as percentages of inhibition of reactive oxygen species at the maximum concentrations tested.

2.4. Antimicrobial activity

The antibacterial potential was assessed against clinical isolates and ATCC strains. The clinical isolates were obtained from patients from the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal) and included five Gram-negative (*Escherichia coli* (VRU12881), *Klebsiella pneumoniae* (VRI17214), *Morganella morganii* (VRU14272), *Proteus mirabilis* (VRI7884) and *Pseudomonas aeruginosa* (VRU14123)) and three Gram-positive strains (*Enterococcus faecalis* (VRU14041), *Listeria*

Table 1
Harvest and processing information of the studied acorn shell samples.

Sample	Species	Harvest origin	Elevation (m)	Geo. coordinates	Harvest date
Q. rot−1	*Q. rotundifolia*	Bragança	660	41°20'08"N 6°46'37"W	2nd half of November 2021
Q. rot−2	*Q. rotundifolia*	Portalegre	159	39°04'35"N 7°55'03"W	2nd half of October 2021
Q. sub−1	*Q. suber*	Braga	331	41°31'43"N 8°18'56"W	2nd half of November 2021
Q. sub−2	*Q. suber*	Braga	331	41°31'43"N 8°18'56"W	1st half of November 2020
Q. rob−1	*Q. robur*	Braga	78	41°32'23"N 8°40'35"W	2nd half of September 2021

monocytogenes (VRU17684) and methicillin-resistant *Staphylococcus aureus* (VRI17654)). The ATCC strains (Liofilchem, Italy) were also tested, including five Gram-negative (*Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica subsp. enterica serovar Enteritidis* (ATCC 13076) and *Yersinia enterocolitica* (ATCC 8610)), and four Gram-positive strains (*Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111), *Staphylococcus aureus* (ATCC 25923) and *Propionibacterium acnes* (ATCC 11827)). The assay was conducted through the microdilution method involving a colorimetric step using *p*-iodonitrotetrazolium chloride (INT) according to the described by de Oliveira et al. (2024). The acorn shell extracts were dissolved using a maximum concentration of 5 % (*v/v*) dimethyl sulfoxide (DMSO) and 95 % autoclaved distilled water to obtain a final concentration of 20 mg/mL for the stock solution that was further serially diluted. The results were expressed as minimum inhibitory (MIC) and bactericidal concentrations (MBC)

Regarding the antifungal capacity, two different strains were used (*Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404), Liofilchem, Italy), following the methodology previously described (Heleno et al., 2013). Fungal spores cultured on malt agar for 72 hours were combined with diluted extract samples in a 96-well microplate. Serial dilutions were carried out, and the plates were incubated at 28 °C for 72 hours. The MIC was determined by direct or microscope visualization of the fungal growth spores, thus evaluating whether there was a growth inhibition effect or not. The minimal fungicidal concentration (MFC) was determined by subculturing the tested compounds for an additional 72 hours at 26 °C. The MFC was identified as the lowest concentration without visible growth, signifying 99.5 % killing of the original inoculum (Heleno et al., 2013).

2.5. Antiproliferative activity

The acorn shell extracts re-dissolved in water (final concentrations tested 6.25–400 μg/mL in water), and the antiproliferative activity was assessed using the colorimetric sulforhodamine B assay, following the protocol described by Mandim et al. (2022). The tested cell lines were routinely maintained with Roswell Park Memorial Institute (RPMI) 1690 medium supplemented with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), heat-inactivated fetal bovine serum (10 %), and non-essential amino acids (2 mM). Cells were maintained at 37 °C with 5 % CO_2 and a humid atmosphere. Cell proliferation was monitored using a phase-contrast microscope, and the culture medium was refreshed approximately every two or three days. The antiproliferative activity was assessed across four human tumor cell lines: gastric carcinoma (AGS), colorectal carcinoma (Caco-2), breast adenocarcinoma (MCF-7), and non-small lung carcinoma (NCI-H460), all commercially acquired from the Leibniz-Institute DSMZ – German Collection of Microorganisms and Cell Cultures GmbH. A non-tumor porcine liver primary culture (PLP2) was also tested. Ellipticine (Sigma-Aldrich, St Louis, MO, USA) was used as the positive control, while a cell solution without samples was used as the negative control. The results were presented as the extract concentration responsible for 50 % of cell proliferation inhibition (GI_{50}, μg/mL).

2.6. NO-production inhibition assay

The extracts capacity to inhibit the production of the pro-inflammatory mediator nitric oxide (NO) in a lipopolysaccharide (LPS)-induced murine macrophage cell line (RAW 264.7) was performed following the methodology previously described by Mandim et al. (2019). Acorn shell extracts were dissolved in water at a final concentration of 8 mg/mL, which was successively diluted to obtain the concentrations to be tested (final concentrations tested 400 – 6.25 μg/mL). The commercial anti-inflammatory dexamethasone (Sigma-Aldrich, Saint Louis, MO, USA) was tested as the positive control, and the cells in the presence and absence of LPS (Sigma-Aldrich, Saint Louis, MO, USA) as the negative control. The results were expressed as the extract concentration responsible for 50 % NO production inhibition (IC_{50}, μg/mL).

2.7. Statistical analysis

The outcomes are presented as the average value along with the mean standard deviation (SD). The data underwent scrutiny through one-way analysis of variance (ANOVA), subsequently subjected to Tukey's HSD test at a significance level of $\alpha = 0.05$ for further examination. This treatment was carried out using RStudio 2023.09.1 + 494.

3. Results and discussion

3.1. Phenolic composition

The obtained chromatographic data and the quantification of the individual compounds detected in the studied acorn shell extracts are shown in Table 2.

A total of nine phenolic compounds were tentatively identified, corresponding to four tannins (peaks **1**, **3**, **4**, **5**) and five phenolic acids (peaks **2**, **6**, **7**, **8**, and **9**). The detected phenolic compounds were tentatively identified as gallic acid (peak **2**) and ellagic acid (peak **8**). Gallic acid derivatives were also identified in the form of either galloyl esters of glucose (peak **3**) or digalloyl esters (peaks **1** and **5**). In addition, ellagic acid derivatives were also detected, being tentatively identified as punicalin (peak **4**), ellagic acid hexoside (peak **6**), ellagic acid pentoside (peak **7**), and methyl ellagic acid pentoside (peak **9**).

Statistically significant differences (significance level <0.01) were observed for all multiple comparisons carried out. The phenolic profile of all samples was quite similar. The most common phenolic compounds in the studied *Quercus* species were gallic and ellagic acids, as well as their derivatives. Although, digalloyl hexoside (peak **1**) being the compound detected in higher concentrations in all samples studied (2.093 – 8.3 mg/g extract), ellagic acid (peak **8**) and its derivatives (peaks **4**, **6**, **7**, and **9**) were also detected in high concentrations. Both were previously identified in acorn extracts (Burlacu et al., 2020; Cantos et al., 2003; Huang et al., 2008; Meyers et al., 2006; Molina-García et al., 2018; Rakić et al., 2006; Vinha et al., 2020) and as compounds present in higher concentrations (Burlacu et al., 2020; Cantos et al., 2003; Molina-García et al., 2018; Rakić et al., 2006; Vinha et al., 2020).

Although the *Q. suber* samples exhibit similar profiles, the *Q. sub-2* sample contains a lower concentration of phenolic compounds compared to *Q. sub-1*. Since the harvest location and soil type for both samples are similar, the observed differences could be related to the harvest year and, therefore, to the known influence that environmental conditions play in the species' secondary metabolism. This hypothesis is further supported by the observed increase in methyl ellagic acid pentoside. Ellagitannin synthesis is often linked to stress responses or adaptation to specific environmental factors (Al-Khayri et al., 2023; Mpofu et al., 2006).

In turn, *Q. rotundifolia* (*Q. rot-1* and *Q. rot-2*) demonstrated a very similar phenolic profile, with *Q. rot-2* exhibiting a higher concentration of gallic acid and derivatives (0.42 – 8.3 mg/g) than *Q. rot-1* (0.90 – 3.9 mg/g). However, the presence of ellagic acid and derivatives is quite similar between the samples studied (1.1927 – 1.259 mg/g and 1.210 – 1.212 mg/g, for *Q. rot-1* and *Q. rot-2*, respectively). The observed differences can be explained by the harvest date and the fruit maturation stage. According to the information described in the literature, oxidation processes can be critical for the decomposition and degradation of phenolic compounds (Alnaizy and Akgerman, 2000; Esplugas et al., 2002; Kumar et al., 2021); therefore, the stage of fruit maturation, harvested, environmental conditions, maintenance, and storage conditions could result in differences in the samples' phenolic composition (Heimler et al., 2017).

Q. robur (*Q. rob-1*) sample exhibits a total phenolic compound content of 9.3 mg/g. This value is in accordance with previous studies

Table 2
Tentative identified phenolic compounds and their quantification (mg/g) in acorn shell extract samples.

Peak	Retention time (min)	λ_{max} (nm)	$[M\text{-}H]^-$ (m/z)	MS^2 (m/z)	Tentative Identification	Quantification (mg/g extract)				
						Q. rot-1	*Q. rot-2*	*Q. sub-1*	*Q. sub-2*	*Q. rob-1*
1	3.32	272	483	169(100)	Digalloyl hexoside	3.9 ± 0.1^{b}	8.3 ± 0.1^{a}	3.38 ± 0.04^{c}	2.93 ± 0.03^{e}	3.16 ± 0.04^{d}
2	4.19	271	169	125(100)	Gallic acid	0.90 ± 0.03^{b}	0.493 ± 0.004^{e}	0.81 ± 0.04^{c}	0.60 ± 0.03^{d}	1.34 ± 0.01^{a}
3	5.06	281	633	463(21),301(100)	Galloyl-HHDP-glucose	tr	0.42 ± 0.01	tr	tr	tr
4	5.32	281	781	781(100),601(22),301(10)	Punicalin	tr	n.d.	n.d.	tr	tr
5	6.96	285	635	465(100), 313(15), 169(6)	Digalloyl-HHDP-hexose	tr	n.d.	tr	n.d.	n.d.
6	9.19	360	463	301(100)	Ellagic acid hexoside	1.1927 ± 0.0003^{c}	n.d.	1.202 ± 0.001^{a}	1.195 ± 0.001^{b}	1.191 ± 0.001^{d}
7	14.33	366	433	301(100)	Ellagic acid pentoside	n.d.	n.d.	n.d.	n.d.	1.193 ± 0.001
8	16.43	359	301	169(100)	Ellagic acid	1.259 ± 0.001^{d}	1.210 ± 0.002^{e}	1.399 ± 0.002^{b}	1.425 ± 0.004^{a}	1.270 ± 0.003^{c}
9	18.48	359	447	301(100)	Methyl ellagic acid pentoside	1.2013 ± 0.0004^{c}	1.212 ± 0.001^{b}	1.217 ± 0.001^{a}	n.d.	1.197 ± 0.001^{d}
					Total Phenolic Compounds	8.5 ± 0.1^{c}	11.6 ± 0.1^{a}	8.0 ± 0.1^{d}	6.15 ± 0.08^{e}	9.3 ± 0.1^{b}

tr – traces; n.d. – not detected. The quantification of the tentatively identified compounds was based on the calibration curves of authentic standards: gallic acid ($y = 131538x + 292163$, $R^2 = 0.9969$; LOD = 8.05 µg/mL and LOQ = 24.41 µg/mL) for compounds 1 – 5; ellagic acid ($y = 26719x - 317255$, $R^2 = 0.9986$; LOD = 0.41 µg/mL and LOQ = 1.22 µg/mL) for compounds 6 – 9. Different letters in the same row correspond to statistically significant differences ($p < 0.05$).

described in the literature that used aqueous extraction. Rakić et al. (2006) characterized aqueous extracts from *Q. robur* pulp and described levels of total phenolic compounds similar to those obtained in the present study.

3.2. Antioxidant, antiproliferative, and NO-production inhibition assays

Table 3 summarizes the results of the antioxidant, antiproliferative, and NO-production inhibition assays.

Regarding lipid peroxidation inhibition by TBARS assay, the *Q. suber* samples (*Q. sub-1* and *Q. sub-2*) exhibited the lowest EC_{50} values and, therefore, the most interesting antioxidant capacity for the TBARS assay. In the case of the acorn shell extract *Q. sub-2*, the obtained values are lower than the positive control used, thus demonstrating its promising ability to inhibit lipid peroxidation. On the other hand, sample *Q. rot-1* exhibits a lower inhibition power, with an EC_{50} value of 18.4 µg/mL. For the cellular antioxidant activity, all samples studied demonstrated the capacity to neutralize the reactive oxygen species and, therefore, to protect cells from oxidative stress. The obtained percentages of inhibition at a concentration of 2 mg/mL of extract comprised between 44 % and 64 %, with sample *Q. rot-1* exhibiting the highest percentage of inhibition. On the other, sample *Q. sub-1* exhibited the lowest capacity to inhibit cellular oxidative stress, with the lowest percentage of inhibition compared to the samples studied. Several studies positively correlate the phenolic composition with the antioxidant activity, which might explain the radical scavenging capabilities demonstrated by the acorn shell extracts for the antioxidant assays tested (Akroum, 2017; Ranjbar Nedamani et al., 2015). The antioxidant properties of acorn shells have not yet been significantly studied. Ferreira et al. (2017) described that *Quercus* acorn flour exhibits an excellent preventive capacity against lipid oxidation and protein carbonylation degradation in chicken patties, describing better lipid peroxidation inhibition values when compared to control patties.

Regarding antiproliferative activity (Table 3), four of the five acorn shell extracts studied demonstrated the capacity to inhibit the proliferation of all tumor cell lines tested. Only the *Q. sub-2* sample showed no activity against the AGS cell line at the concentrations tested (GI_{50} >400 µg/mL). Although *Q. sub-2* extract did not exhibit the capacity to interfere with the proliferation of the AGS cell line, it was the sample that demonstrated the highest antiproliferative activity for the remaining cell lines tested, with GI_{50} values lower than 200 µg/mL. For the non-tumor PLP2 cell line, *Q. rot-2* exhibited the highest GI_{50} values and, therefore, higher cytocompatibility for liver cells among the tested extracts. Several works identify quercetin, myricetin, kaempferol and its glycosides, and ellagic acid as the essential anticancer agents detected in *Quercus* species (Baliga et al., 2019; Cui et al., 2008; Sultana and Anwar, 2008). According to Moradi et al. (2016), the antiproliferative effect of crude extract from acorns inhibits tumor cell proliferation by inducing early apoptosis in AGS cell lines, which is in agreement with the results obtained in our work.

Regarding the NO-production inhibition (Table 3), none of the samples studied demonstrated the capacity to inhibit the NO production at the tested concentrations (IC_{50} >400 µg/mL). Several studies have demonstrated the anti-inflammatory capacity of different species of acorn extracts (Burlacu et al., 2020; Castejón et al., 2019; Chokpaisarn et al., 2017). However, these studies used the acorn pulp instead of the shell, which may justify the observed differences.

3.3. Antimicrobial activity

Table 4 presents the results regarding the antimicrobial activity of the studied acorn shell extracts against clinical and foodborne bacterial strains, as well as fungal strains.

Most of the tested extracts demonstrated the capacity to inhibit bacterial growth, however, no antifungal activity was observed for the fungi tested. Sample *Q. sub-1* exhibits the most promising activity

Table 3
Results of antioxidant (TBARS and CAA assays), antiproliferative, and NO-production inhibition assays of the studied acorn shell extracts.

	Q. rot-1	*Q. rot-2*	*Q. sub-1*	*Q. sub-2*	*Q. rob-1*	Positive Control
Antioxidant Activity						
TBARS (EC$_{50}$, μg/mL)	18.4 ± 0.1^{a}	14.0 ± 0.1^{b}	6.02 ± 0.04^{c}	4.0 ± 0.2^{d}	13.4 ± 0.1^{b}	5.39 ± 0.28
CAA (% inhibition at 2 mg/mL)	64 ± 8	49 ± 9	44 ± 9	56 ± 10	57 ± 7	95 ± 5
Antiproliferative activity (GI$_{50}$, μg/mL)						
AGS	195 ± 16^{a}	162 ± 12^{b}	138 ± 13^{c}	> 400	167 ± 15^{b}	1.23 ± 0.03
Caco−2	213 ± 18^{a}	183.34 ± 19^{b,c}	162.05 ± 7,42^{c}	164 ± 15^{c}	206 ± 4^{a,b}	1.21 ± 0.02
MCF−7	286 ± 51^{a}	223 ± 13^{a,b,c}	210 ± 6^{b,c}	181 ± 32^{c}	271 ± 55^{a,b}	1.02 ± 0.02
NCI-H460	278 ± 23^{a}	201 ± 19^{b}	207 ± 18^{b}	153 ± 12^{c}	251 ± 22^{a}	1.01 ± 0.01
PLP2	252 ± 22^{a}	265 ± 27^{a}	191 ± 18^{b}	134 ± 36^{c}	248 ± 28^{a}	1.4 ± 0.1
NO-production inhibition (IC$_{50}$, μg/mL)						
RAW 264.7	> 400	> 400	> 400	> 400	> 400	6.3 ± 0.4

EC$_{50}$ – half maximal effective concentration; GI$_{50}$ – concentration responsible for 50 % cell proliferation inhibition; IC$_{50}$ – extract concentration needed to provide 50 % inhibition in nitric oxide (NO) production; AGS – gastric adenocarcinoma; Caco-2 – colorectal adenocarcinoma; MCF-7 – breast adenocarcinoma; NCI-H460 – lung carcinoma; PLP2 – porcine liver primary cells; RAW 264.7 – murine macrophage cells. Positive controls: TBARS - Trolox; CAA: Quercetin (% inhibition at 0.3 μg/mL); antiproliferative activity - Ellipticine; NO-production inhibition - Dexamethasone. Different letters in the same row correspond to statistically significant differences ($p < 0.05$).

Table 4
Antimicrobial activity of acorn shell extracts.

		Acorn shell samples					Positive Controls		
		Q. rot-1	*Q. rot-2*	*Q. sub-1*	*Q. sub-2*	*Q. rob-1*			
Antibacterial activity - Clinical bacteria (MIC/MBC, mg/mL)									
							Ampicillin	Imipenem	Vancomycin
Gram-negative	*E. coli*	10/10	> 10/> 10	2.5/10	> 10/> 10	> 10/> 10	< 0.15/< 0.15	< 0.0078/< 0.0078	n.t./n.t.
	K. pneumoniae	10/> 10	> 10/> 10	5/> 10	> 10/> 10	> 10/> 10	10/> 10	< 0.0078/< 0.0078	n.t./n.t.
	M. morganii	2.5/2.5	> 10/> 10	0.3/1.25	> 10/> 10	> 10/> 10	> 10/> 10	< 0.0078/< 0.0078	n.t./n.t.
	P. mirabilis	2.5/10	> 10/> 10	2.5/10	> 10/> 10	> 10/> 10	< 0.15/< 0.15	< 0.0078/< 0.0078	n.t./n.t.
	P. aeruginosa	10/> 10	> 10/> 10	1.25/> 10	> 10/> 10	> 10/> 10	> 10/> 10	0.5/1	n.t./n.t.
Gram-positive	*E. faecalis*	10/10	> 10/> 10	10/> 10	> 10/> 10	> 10/> 10	< 0.15/< 0.15	n.t./n.t.	< 0.0078/< 0.0078
	L. monocytogenes	10/> 10	> 10/> 10	2.5/10	> 10/> 10	> 10/> 10	< 0.15/< 0.15	< 0.0078/< 0.0078	n.t./n.t.
	*MRSA**	1.25/10	> 10/> 10	0.6/10	> 10/> 10	> 10/> 10	< 0.15/< 0.15	n.t./n.t.	0.25/0.5
	P. acnes	2.5/10	> 10/> 10	2.5/> 10	> 10/> 10	> 10/> 10	0.07/0.07	n.t./n.t.	n.t./n.t.
Antibacterial activity - Food bacteria (MIC/MBC, mg/mL)									
							Ampicillin	Streptomycin	Methicillin
Gram-negative	*E. cloacae*	5/> 10	> 10/> 10	2.5/2.5	> 10/> 10	> 10/> 10	0.15/0.15	0.007/0.007	n.t./n.t.
	E. coli	10/10	> 10/> 10	5/10	> 10/> 10	> 10/> 10	0.15/0.15	0.01/0.01	n.t./n.t.
	P. aeruginosa	10/> 10	> 10/> 10	10/> 10	> 10/> 10	> 10/> 10	0.63/0.63	0.06/0.06	n.t./n.t.
	S. enterica	5/10	> 10/> 10	2.5/5	> 10/> 10	> 10/> 10	0.15/0.15	0.007/0.007	n.t./n.t.
	Y. enterocolitica	10/10	> 10/> 10	5/5	> 10/> 10	> 10/> 10	0.15/0.15	0.007/0.007	n.t./n.t.
Gram-positive	*B. cereus*	> 10/> 10	> 10/> 10	10/10	> 10/> 10	> 10/> 10	n.t/n.t	0.007/0.007	n.t./n.t.
	L. monocytogenes	10/> 10	> 10/> 10	2.5/5	> 10/> 10	> 10/> 10	0.15/0.15	0.007/0.007	n.t./n.t.
	S. aureus	2.5/> 10	1.25/> 10	0.3/5	1.25/> 10	> 10/> 10	0.15/0.15	0.007/0.007	0.007/0.007
Antifungal activity (MIC/MFC, mg/mL)									
							Ketoconazole		
	A. brasiliensis	> 10/> 10	> 10/> 10	> 10/> 10	> 10/> 10	> 10/> 10	0.06/0.125		
	A. fumigatus	> 10/> 10	> 10/> 10	> 10/> 10	> 10/> 10	> 10/> 10	0.5/1		

n.t. – Not tested; * Methicillin-resistant *Staphylococcus aureus.* MIC – minimal inhibitory concentration; MBC – minimal bactericidal concentration; MFC – minimal fungicidal concentration. Positive controls: Ampicillin, Imipenem, Vancomycin, Streptomycin, Methicillin, and Ketoconazole.

against both clinical and food bacteria, with the lowest MIC (between 0.3 and 10 mg/mL) and MBC values (between 1.25 and >10 mg/mL). When compared to the positive controls tested, namely ampicillin, this extract exhibits a more promising activity, with lower MIC values against the clinical bacteria *K. pneumoniae* and *M. morganii*. For the other, samples *Q. rot-2*, *Q.sub-2*, and *Q.rob*-1, exhibited antibacterial activity only towards the food bacteria *S. aureus* (MIC = 1.25 mg/mL).

Oak bark has been recognized and utilized for its medicinal properties since ancient times. Due to its antimicrobial properties, it was commonly topically applied to treat burns and wounds and to prevent infections (Hemingway and Laks, 2012). Nevertheless, this potential has also been demonstrated by scientific studies. Silva et al. (2023), demonstrated the antimicrobial potential of *Quercus ilex* and *Q. suber* acorn extracts, particularly against Gram-positive bacteria such as methicillin-resistant *S. aureus*, *B. cereus,* and *L. monocytogenes*. These results highlight the potential application of acorn by-products as natural preservatives or antimicrobial agents.

4. Conclusions

This study highlights the significant bioactive potential of acorn shell extracts, particularly in relation to their phenolic composition, antioxidant capacity, and antimicrobial and antiproliferative activities. In total, nine phenolic compounds were tentatively identified, predominantly gallic acid, ellagic acid, and their derivatives, with digalloyl hexoside being the most abundant across all samples. The phenolic profile varied between species and regions, underscoring the influence of environmental and processing conditions on the chemical composition. The samples studied exhibit antioxidant potential, with *Q. suber* harvested in Braga (*Q. sub-2*) exhibiting a higher capacity for lipid peroxidation inhibition than the commercial antioxidant compound tested as a positive control. In terms of antiproliferative activity, the extracts inhibited the growth of various human tumor cell lines, with *Q. suber* samples showing the most promising effects. This suggests potential applications in cancer prevention or adjunct therapies, warranting further exploration into the mechanisms of action and specific bioactive compounds responsible for these effects. Antimicrobial analysis revealed significant antibacterial potential against both clinical and foodborne pathogens, particularly demonstrated by the *Q. sub-1* extract, which displayed noteworthy activity against resistant strains like *Morganella morganii*, MRSA, and *Staphylococcus aureus*.

None of the samples studied exhibit antifungal properties and the capacity to inhibit the production of pro-inflammatory mediator nitric oxide.

Overall, the study underscores the unexploited potential of acorn shells as a sustainable source of phenolic compounds with diverse bioactivities. These findings pave the way for further research aimed at isolating active constituents, optimizing extraction methods, and exploring acorn extracts' practical applications in functional foods, pharmaceuticals, and antimicrobial solutions. The development of further studies with a larger number of samples in order to obtain more detailed information and, therefore, identify the bioactive compounds responsible for the promising activities demonstrated would be of great interest.

CRediT authorship contribution statement

Mateus Cristiano: Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Alonso-Esteban Ignacio:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Finimundy Tiane:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Mandim Filipa:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Oliveira Izamara:** Validation, Methodology, Investigation, Data curation. **Babo Pedro:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Canadas Raphaël:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Ferreira Isabel:** Writing – review & editing, Supervision. **Barros Lillian:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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