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Red Oak (*Quercus rubra* L.) Fruits as Potential Alternative for Cocoa Powder: Optimization of Roasting Conditions, Antioxidant, and Biological Properties

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Abstract: Cocoa powder is a basic ingredient in the manufacture of chocolate, one of the most appreciated sweet products in the world for its sensory and nutritional properties. Furthermore, it displays a central nervous system stimulant effect. This study aimed to investigate acorn-derived powder as an alternative to cocoa powder, in order to obtain a chocolate that does not contain stimulants of the nervous system. Both the chocolate technological process and acorns roasting process (180 °C/25 min, 200 °C/20 min and 220 °C/15 min) were optimized to obtain acorn powder with an organoleptic profile as close as possible to that of cocoa powder. The chocolate sensory evaluation was performed by means of the hedonic test. Furthermore, the aqueous extracts obtained from the resulting powder were evaluated for total polyphenol content, and in vitro antimicrobial and antiproliferative properties. The results point out a high content of phenolic compounds (500.78–524.01 mg GAE/100 g); protection against microbial contamination based on the ability to inhibit a Gram-positive bacterium (*Bacillus cereus*) was also noticed. The aqueous acorn extracts were also able to reduce the cell viability of HFL-1 (human fetal lung fibroblast) and DLD-1 (colorectal adenocarcinoma) lines. This study suggests red oak (*Quercus rubra*) fruits as a potential alternative to cocoa powder in the manufacture of chocolate.

Keywords: acorn; polyphenols; antibacterial activity; antiproliferative activity; chocolate

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1. Introduction

Cocoa is the main ingredient in the manufacture of chocolate products, the most popular and best-selling sweet food in the world. Cocoa-based products are appreciated for their sensoriality, special aroma and pleasant melt-in-the-mouth properties [1–3].

Numerous studies have shown the importance of cocoa consumption to promote health and prevent or treat diseases, by mechanisms such as oxidative stress reduction, antioxidant properties, cardiovascular protective activity, inhibition of LDL (low-density lipoprotein) oxidation or even cancer [4]. In addition to the advantages presented above, cocoa has stimulants, which in many situations are undesirable. These substances, called purine alkaloids, such as caffeine, theobromine and theophylline, are substances that act on the nervous system, and include effects such as increased concentration and attention and vasodilation with a consistent increase in diuresis [5].

For this reason, attempts were made to search for alternatives to cocoa powder. The most common substitute is carob powder. Carob (*Ceratonia siliqua*) derives from the Greek keras (horn) and the Latin *siliqua*, alluding to the hardness and shape of the pod. It is

also called St. John's bread or locust bean, because it was supposedly used as food by St. John the Baptist. In recent years, the demand for carob has increased due to the bioactive components it contains; it is a powerful antioxidant and is rich in minerals. It contains no nervous system stimulants and is successfully used as a cocoa substitute [4,6]. It has been successfully used as a cocoa substitute (partially or totally) in the manufacture of milk chocolate. There were reductions in sugar content, a slight improvement in protein content, an increase in potassium, calcium, sodium and magnesium, but also a decrease in iron and zinc levels. The most notable effect was a decrease in caffeine, while at the same time the sensory properties were not affected [7]. Other dishes in which cocoa powder was successfully replaced with carob powder include gluten-free soy and banana cakes [8], carob-based milk drink that can provide an alternative to chocolate milk [9] or low-fat yogurt with a chocolate-like taste [10].

Besides carob powder, acorns might be another alternative for substituting cocoa powder in the manufacture of chocolate. Acorn is the fruit of the oak, and is part of the genus *Quercus*, which includes about 600 species worldwide. It has been popular for thousands of years in the cuisine of many countries, nations and cultures [11]. They were considered a staple food in Spain [12], Italy (Sardinia) [13], Turkey [14], Poland [15], Asian countries and Central, East and North America. Oak fruits are eaten raw, boiled or fried to make oil, bread, cakes, porridge, soup or coffee-like beverages [13,16], or used in ice cream, desserts or liqueurs [17,18] and coffee [13,19,20].

In terms of composition, acorns have compounds comparable to those of cereals, being a rich source of carbohydrates, predominantly starch, with levels between 31 and 51% depending on the species. They also contain protein between 2 and 8% and fat between 0.7 and 9% [18,21–23]. For this reason, most studies present acorns as a suitable component for the bakery and pastry industry, in items such as acorn bread [22,24,25], biscuits [26], muffins [27] or cake [28].

Even though acorns have a high content of tannins, which give a bitter taste [15,16], acorn flour, due to the production process, does not contain large amounts of anti-nutritional substances. It is a valuable source of magnesium, calcium, potassium, iron, copper, zinc, manganese [29], B vitamins [30], dietary fiber [31], tocopherols [21,32], unsaturated fatty acids (60% oleic acid, ω9 and 16% linoleic acid, ω6) [33,34], chlorophylls, carotenoids and antioxidants (especially polyphenols) [34,35].

In addition to the antioxidant effect, several studies have shown that acorns present varying in vitro antibacterial efficacy against *Staphylococcus aureus*, *Enterobacter aerogenes*, *Bacillus subtilis*, *E. coli*, *Klebsiella pneumoniae* and *Staphylococcus epidermidis*, an antifungal effect against *Candida albicans* [36–39] and also an antiproliferative effect [40–42]. The antioxidant and antimicrobial activity differs depending on the type of acorn and the type of extract used.

Based on the previously discussed and reported uses of acorns, it is important to highlight the novelty of the current paper, which aims to substitute cocoa powder with acorn powder, while obtaining an innovative product that does not contain stimulants of the nervous system. Furthermore, the aqueous extracts obtained from the resulting acorn powder were evaluated for total polyphenol content, and in vitro antimicrobial and antiproliferative properties.

2. Materials and Methods

2.1. Materials

The acorns of the red oak (*Quercus rubra* L.) were harvested manually from the campus of the University of Agricultural Sciences and Veterinary Medicine (UASVM) Cluj-Napoca during October and November.

The acorns were collected at maturity when their color was yellow-brown or brown, being crossed by dark brown stripes, and when the size of the oak fruits reached on average 2–4 cm in length and 1–1.5 cm in diameter. After harvesting, the acorns were subjected to the conditioning operation. Conditioning aims to free oak fruits from impurities (soil,

leaves, cups) and sort them to remove moldy, broken, cracked acorns, attacked by animals and diseases.

The roasting was performed at different time and temperature intervals to highlight the aroma and to obtain organoleptic characteristics as close as possible to those of cocoa powder. During the roasting process, the temperature in the room was 22–23 °C and the relative humidity was 40–42%. After grinding the roasted acorn kernels, three types of powders were obtained, heat treated differently. Figure 1a shows the powder obtained from heat-treated acorns at a temperature of 180 °C for 25 min, Figure 1b at 200 °C for 20 min and Figure 1c at 220 °C for 10 min, respectively.

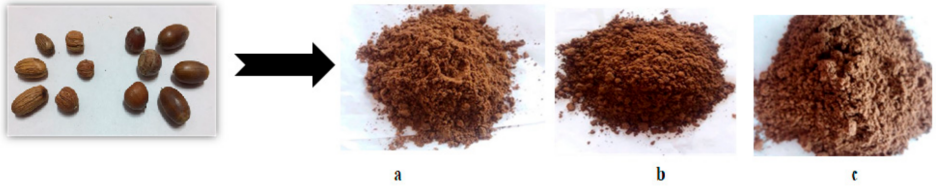

Figure 1. Acorn powder obtained from heat-treated acorns at 180 °C/25 min (**a**), 200 °C/20 min (**b**) and 220 °C/10 min (**c**).

The amount of acorn powder used for chocolate manufacturing was relatively low due to its more intense flavor (more astringent) as compared to cocoa powder; roasted acorn powder, when added to white chocolate mass in higher concentrations (>10%), completely changes the taste to which consumers are accustomed. For this reason, 7% acorn powder addition was established empirically based on preliminary sensory tests. The chocolate samples were obtained by heating the white chocolate mass to 40–42 °C, and after complete melting, the acorn powder was incorporated into this mass by continuous homogenization until a homogeneous mixture was obtained. Then, the chocolate samples were subjected to the tempering operation, while cooling the mass to 27–28 °C and then re-heating to 30–31 °C, and poured into molds. Tempering was carried out in order to obtain a chocolate with a glossy surface and to facilitate the unmolding. Finally, the chocolate samples were packed and stored till further sensory analysis was performed.

2.2. Determination of Total Polyphenol Content by Folin–Ciocâlteu Method

The total polyphenols content was assessed using the Folin–Ciocâlteu method [43], slightly modified. Extraction of oak fruit samples was performed from 1 g of sample (acorn powder) over which methanol (0.01% HCl) was added and the sample was centrifuged, followed by successive extractions, filtration, rotary evaporation, recovery in methanol (10 mL) and storage at −20 °C until analysis of polyphenols. A quantity of 25 µL sample (extract) was mixed with 1.8 mL of distilled water and 120 µL Folin–Ciocâlteu reagent in a glass vial. Na_2CO_3 7.5% solution in distilled water (340 µL) was added 5 min later, to assure basic conditions (pH 10) for the redox reaction between the phenolic compounds and the Folin–Ciocâlteu reagent. The samples were incubated for 90 min at room temperature. Methanol was used as a control sample. The absorbance at 750 nm was measured using a Shimadzu UV-VIS 1700 spectrophotometer. The calibration curve was plotted based on the 0.25, 0.50, 0.75 and 1 mg mL^{-1} concentrations of gallic acid. The total polyphenol content was expressed in gallic acid equivalents (GAE)-mg GAE 100 g^{-1} [44].

2.3. Microbiological Analyses

The in vitro antibacterial potential of aqueous extracts was evaluated against the following reference strains: *Staphylococcus aureus* ATCC® 6538P, *Bacillus cereus* ATCC® 14579, *Escherichia coli* ATCC® 25922, *Salmonella enteritidis* ATCC® 13076 and *Salmonella typhimurium* ATCC® 14028.

Microbial strains of 18–24 h were used to prepare the inoculum; 4–6 identical colonies were suspended in sterile conditions to obtain the inoculum. The size of the inoculum was standardized on a nephelometric basis, using the McFarland scale. For antibacterial

screening tests in the case of plant extracts, different bibliographic sources indicate different values of the bacterial inoculum.

In the case of the present study, the bacterial inoculum used was Inoculum (1)—number 0.5 on the McFarland scale (turbidity corresponds to a concentration of approximately 1.5×10^8 CFU/mL); the value of 0.5 is indicated for in vitro susceptibility testing of synthetic antimicrobial agents. The bacterial inoculum was evenly distributed on Mueller Hinton agar. The plates were kept in the vicinity of a gas bulb for 10–15 min for blasting, then wells were stamped and the products included in the test were distributed (code 1–30) (2 wells/sample with duplicate testing).

The reading of the results was performed after 24 h of incubation at 37 °C and consisted of assessing the size of the induced inhibition zones, areas where microbial colonies were missing. The results are expressed in the form of the average values obtained by performing the arithmetic mean of the diameters corresponding to the 2 tests [45,46].

2.4. Determination of Cell Viability

HFL-1 (human fetal lung fibroblast) and DLD-1 cell lines (colorectal adenocarcinoma) were used to test the aqueous extracts. The HFL-1 cell line was used in step 18. After thawing, it was cultured in DMEM/F12 propagation medium (Gibco) supplemented with 10% FCS (fetal calf serum, Sigma-Aldrich), 1% NEA (non-essential amino acids, Sigma-Aldrich) and 1% antibiotics–antimycotics (Gibco). The DLD1 line was used in passage 31. The cell line was cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (Sigma-Aldrich) and 1% antibiotics–antimycotics (Gibco).

The tests were performed on 96-well plates, and the cell concentration was 1×10^5. After 24 h of incubation, the cell cultures were treated with the selected products, and after 24 h of exposure the cell viability was determined by the MTT test.

The MTT test is based on the detection of the reduction of a chemical compound called MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide) by mitochondrial dehydrogenase, with the formation of formazane, a blue product. The process reflects the normal functioning of mitochondria and thus cell viability. After removal of the culture medium, the cells were treated with 100 µL MTT solution (0.5 mg MTT/mL/buffer/HBSS) and incubated for 3 h at 37 °C. Subsequently, the MTT reagent was removed and 100 µL of dimethylsulfoxide (DMSO) (Sigma) was distributed in each well, which had the role of solubilizing the formazan particles, after which the spectrophotometric reading of the optical densities was performed with a microplate reader for the length of 450 nm [47].

The results obtained from the MTT test (optical densities for each product and the diluent used) were processed as mean ± standard deviation and subsequently presented as a percentage of viability (%) obtained by reporting the average value of optical densities recorded for each product tested at average optical density determined for control (untreated cells, cultured under the same standard conditions):

$$\% \text{ viability} = \frac{\text{DO average sample}}{\text{DO average control sample}} \times 100$$

where DO—optical density.

The intensity of the expression of the cytotoxic potential was assessed by statistical comparison of the viability percentages obtained for each product at 100% (% viability of the control sample).

2.5. Sensory Evaluation

The sensory evaluation was performed by means of the hedonic test according to ISO 13299:2016. Sensory characteristics of samples were evaluated by 50 trained panelists (31 female and 19 male), aged between 18 and 63. The degree of pleasure for chocolate with cocoa powder and chocolate with acorn powder was rated based on a 9-point hedonic scale (1 being "extreme dislike" and 9 being "greatly like"). Overall acceptability, smell,

color, texture, appearance and taste were the sensory attributes that were evaluated. Water was used to rinse the mouth before and after each test [48].

2.6. Statistical Analysis

All analyses were performed in duplicate replications, using MINITAB software. Differences were analyzed using one-way analysis of variance ANOVA (analysis of variance). Significance of differences between means for each parameter was determined by Tukey's test at a significance level of $p < 0.05$. All results are presented as mean ± SD (standard deviation).

3. Results and Discussion

3.1. Optimization of Roasting for Acorn Powder Production

Given that the organoleptic profile of the acorns was unknown for the present study, the temperature setting for the heat treatment was started at 135 °C with a minimum duration of 5 min, but there was no visible change (Table 1). For this reason, the extension of the roasting time was continued until 90 min; however, even at this threshold, only positive color changes were registered, the flavor profile being quite poorly defined. Due to the fact that the roasting time at this temperature was too long and economically a disadvantage, the following trials were performed at 160 °C for 10, 20, 30, 45, 60, 75 and 90 min; after 30 min, partially favorable organoleptic changes were noticed.

Table 1. Organoleptic profile of roasted acorns at 135 and 160 °C at different time intervals.

Time (min)	Temperature (°C)			
	135 °C ± 5 °C		160 °C ± 5 °C	
	Color	Flavor	Color	Flavor
5	unchanged	without flavor	unchanged	without flavor
10			light brown	without flavor
20	unchanged	without flavor	slight dark brown	without flavor
30	unchanged	without flavor	slight dark brown	without flavor
45				
60				
75	slight dark brown	slight flavor	very dark brown	well flavor
90	dark brown	slight flavor	very dark brown	strong flavor

Table 1 shows the organoleptic profile of roasted acorns at temperatures of 135 °C and 160 °C, at different time intervals. According to the results in the table, as the temperature increases, the roasting time decreases and the organoleptic profile changes take place much faster.

In order to obtain the desired organoleptic profile in the shortest possible time, the roasting was performed also at 180, 200 and 220 °C. The differences observed at these temperatures in terms of color and aroma are shown in Tables 2 and 3.

According to the results presented in Table 2, the choice of the optimal parameters for roasting, if the aim is only to obtain a color as close as possible to that of roasted cocoa beans, is obvious (220 °C, 10 min), but in terms of flavor profile, things are completely different. Thus, at these parameters, although the color is very well formed, the aroma is not completely defined.

In brief, analyzing all studied time–temperature parameters, the most suitable for roasting acorns were identified as 180 °C/25 min, 200 °C/20 min and 220 °C/15 min. However, to obtain chocolate, it was proposed to roast the acorns at a temperature of 200 °C for 20 min, which seems to provide the acorns with an aroma and color profile closest to cocoa powder.

Table 2. Variation in the color of roasted acorns at 180, 200 and 220 °C at different time intervals.

Time (min)	Temperature (°C)		
	180 °C ± 5 °C	**200 °C ± 5 °C**	**220 °C ± 5 °C**
5	unchanged	unchanged	dark brown
10	light brown	light brown	**very dark brown**
15		dark brown	
20	dark brown	**very dark brown**	black
25	**very dark brown**		

Table 3. Variation in the flavor of roasted acorns at 180, 200 and 220 °C at different time intervals.

Time (min)	Temperature (°C)		
	180 °C ± 5 °C	**200 °C ± 5 °C**	**220 °C ± 5 °C**
5	without flavor	without flavor	slightly flavor
10		slight flavor	well flavor
15	slight flavor	well flavor	**strong flavor**
20	well flavor	**strong flavor**	charred
25	**strong flavor**	charred	

3.2. Total Polyphenol Content of Roasted Acorn Powder

As can be seen in Table 4, the highest total polyphenol content was recorded in the heat-treated acorn at 180 °C for 25 min with a value of 524.01 mg GAE/100 g sample. Thus, the heat-treated acorn at 180 °C/25 min has a significantly higher total polyphenol content compared to raw acorns. This is due to the fact that during the heat treatment, the tannins that are found in a fairly high amount in the raw acorn degrade, and thus increase the content of non-tannin phenolic compounds, especially gallic acid.

Table 4. The influence of heat treatment on the total polyphenol content of acorn aqueous extracts.

Samples	Total Polyphenol Content (mg GAE/100 g)
Raw acorn	$500.78^{b} \pm 0.87$
Roasted acorn at 180 °C	$524.01^{a} \pm 1.27$
Roasted acorn at 200 °C	$519.20^{a} \pm 1.07$
Roasted acorn at 220 °C	$521.83^{a} \pm 1.75$

Identical superscript letters indicate no significant difference ($p > 0.05$).

In order to obtain a more complete view, the results obtained were correlated with those obtained by Youn et al., who obtained values between 375.96 (water extract) and 288.01 mg GAE/g (methanol extract) for acorns of *Quercus acutissima* Carruth [49]. Ranjbar Nedamani et al. obtained different results for oak fruits (*Quercus branti*) of 22.64 g of gallic acid per 100 g (methanol extract / dry weight) [50] compared to us, and Khanav et al. obtained results of up to 88.43 GAE/100 g (water:methanol extract) [51]. Results very close to those reported by us were reported by Zhou et al.: 512.68 mg GAE/g in the case of aqueous extracts of *Quercus variabilis* Blume (*Fagaceae*) [52].

Rakic et al. recorded values of the total polyphenol content of 12.33% for raw acorns, 11.76% for heat-treated acorns at 200 °C for 15 min and 14.93% for aqueous extract obtained from heat-treated acorns [53]. Thus, the results obtained by them indicate that acorns are a raw material rich in polyphenols and that once the tannins are degraded during heat treatment there is an increase in the content of polyphenols, and also the heat-treated samples have a higher antioxidant capacity than raw material.

Given the results of previous studies and correlations with specialized studies, the basic idea is that after heat treatment there is an increase in total polyphenols; however,

there is still a result that does not meet this expectation, namely the result of the heat-treated sample at 200 °C/20 min, which recorded a slightly lower value than at 180 °C, thus concluding that the differences may be due to the roasting time and temperature, the different extraction procedures and the differences between the fruits at ripening.

3.3. The Antimicrobial Potential of Acorn Aqueous Extracts

In vitro antibacterial potential of aqueous acorn extracts was evaluated against the following reference strains: *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Salmonella enteritidis*, *Salmonella typhimurium*.

The obtained results presented in Table 5 represent the appreciation of the size of the inhibition zones compared to the reference strains, obtained by performing the arithmetic mean of the corresponding diameters of 2 tests.

Table 5. Diameter of inhibition zone (mm) determined against the reference strains.

Sample	*Salmonella enteritidis*	*Salmonella typhimurium*	*Staphylococcus aureus*	*Bacillus cereus*
Aqueous extract of roasted acorn 220 °C	n.d.	n.d.	n.d.	$12^{b} \pm 0.00$
Aqueous extract of roasted acorn 200 °C	n.d.	n.d.	n.d.	$11.5^{b} \pm 0.71$
Aqueous extract of roasted acorn 180 °C	n.d.	n.d.	n.d.	$11^{b} \pm 0.00$
Aqueous raw acorn extract	n.d.	n.d.	n.d.	$14.5^{a} \pm 0.71$
Control-distilled water	n.d.	n.d.	n.d.	n.d.

Identical superscript letters indicate no significant difference ($p > 0.05$); n.d. = not detected.

Aqueous acorn extracts did not display an antibacterial effect against Gram-negative bacteria (*Salmonella enteritidis* ATCC® 13076, *Salmonella typhimurium* ATCC® 14028), but presented inhibitory properties against a Gram-positive species, namely *Bacillus cereus* ATCC® 14579. The most intense inhibitory effects against this reference strain were observed for the aqueous extract of raw acorns (not heat treated), recording a value of 14.5 mm for the inhibition zone diameter and for the aqueous extract of acorn fried at 220 °C with a value of 12 mm. The negative result for the control antimicrobial effect, distilled water, indicates the effectiveness of aqueous acorn extracts.

Aqueous acorn extracts possess both antioxidant and antimicrobial capacity due to the high content of total polyphenols (especially tannins). According to Sung et al., similar to other polyphenols, tannins display antioxidant and antimicrobial properties, which are mainly justified by their ability to inhibit hydrolytic enzymes (proteases and carbohydrolases) and to bind transport proteins, thus preventing microbial contamination [38].

In addition, Andrensek et al. also suggested that oak (*Quercus robur* L.) could be a natural plant material with antimicrobial and antioxidant activities. The antimicrobial activity has been described for multi-step elution of extracts with different solvents in a ExtraChromR multifunctional instrument. Interestingly, the extracts obtained with the mixture methanol:ethyl acetate 50:50 (*v*/*v*) and methanol:water 75:25 (*v*/*v*) showed inhibitory potential against *S. aureus*, but the less polar extracts (acetate ethyl acetate:hexane 75:25 and ethyl acetate:methanol 95:5) were particularly active against one Gram-negative bacterium (*Enterobacter aerogenes*) and against fungi (*Candida albicans*) [37].

Uddin and Rauf evaluated the bioactive compounds content in the aerial parts of *Quercus robur* L. and associated different phytochemical classes with antimicrobial and antioxidant activities. For antimicrobial activity, n-hexane, ethyl acetate, chloroform and methanolic fractions of *Quercus robur* were evaluated against five different bacteria (*B. subtilis*, *E. coli*, *K. pneumoniae*, *S. aureus* and *S. epidermidis*), showing different activities [36].

Moderate antibacterial and fungal activity for *B. subtilis*, *E. coli*, *K. pneumonia* and *S. aureus* was reported by Ahmed et al. for *Quercus floribunda* L. [41], while Sati et al. reported antimicrobial activity towards *B. subtilis*, *S. aureus*, *P. aeruginosa* and *E. coli* for an extract obtained from *Quercus leucotrichophora* fruits [54].

Thus, our results are in agreement with previously published studies [36,37,41,54], highlighting the acorn extract's antimicrobial potential. The inhibitory properties against Gram-positive and Gram-negative bacteria appear to be influenced by the chosen reference strains, the manner in which the extract was obtained and whether or not the raw material was subjected to heat treatment.

3.4. The Influence of Acorn Aqueous Extracts on Cell Viability

HFL-1 (human fetal long fibroblast) and DLD-1 (colorectal adenocarcinoma) cell lines were used to perform antiproliferative assays on selected extracts.

Table 6 shows the results on the cell viability of aqueous acorn extracts on the DLD-1 cancer line.

Table 6. Cellular viability of the DLD-1 line.

		Aqueous Roasted Acorn Extract			
	Absolute Control	**180 °C**	**200 °C**	**220 °C**	**Raw**
OD450	0.765	0.241	0.201	0.176	0.306
OD450	0.721	0.267	0.317	0.169	0.367
Arithmetic mean	0.743	0.254	0.259	0.1725	0.3365
Viability	100%	34.19	34.86	23.22	45.29

Following the results obtained, it is observed that the cell viability for the DLD-1 cancer line decreased considerably in the case of the aqueous extract obtained from the heat-treated acorn at 220 °C, registering a viability of 23.22% compared to the absolute control. According to Vinha et al., this is due to the fact that acorns have a high content of bioactive compounds that have many biological functions, such as antitumor, antiallergic, antithrombotic, anti-ischemic and anti-inflammatory activities [35].

After the heat-treated acorn at 220 °C, the aqueous extract obtained from the heat-treated acorn at 180 °C with a viability of 34.19% follows, then that of the heat-treated acorn at 200 °C with a viability of 34.86% and, finally, the aqueous extract obtained from raw acorns with a viability of 45.29%. The reason why the results obtained for the aqueous extracts from the acorn that was subjected to the heat treatment, compared to the results obtained for the raw acorn extract, register a better stopping of cell viability is that after the heat treatment, the tannins degrade, leading to the increased polyphenol content, antioxidant capacity and thus antiproliferative activity.

Studies by Seeram et al. concluded that blueberries are an excellent source of phytochemicals that include: glycosides, flavonoids, anthocyanins, proanthocyanids (condensed tannins) and organic and phenolic acids. They performed anti-proliferative tests on various cell lines, including colon cancer cells (HT-29, HCT116, SW480, SW620), obtaining a higher antiproliferative activity against HCT116 (92.1%) than against HT-29 (61.1%), SW480 (60%) and SW620 (63%) [55].

Observing the correlation between the results obtained on aqueous acorn extracts and those obtained by Seeram et al. [55] on blueberry extracts, it can be stated that the two fruits have an increased antiproliferative activity due to the high content of biologically active compounds.

The way in which aqueous acorn extracts act on the viability of DLD-1 cells is also shown in Figure 2, where the differences mentioned above can be observed.

Table 7 shows the results obtained on the cell viability of aqueous acorn extracts on the HFL-1 line. Following the results obtained, it is observed that the cell viability for the HFL-1 line decreased the most in the case of the aqueous extract obtained from the heat-treated acorn at 220 °C, as in the previous case, registering a viability of 75.5% compared to the control absolute.

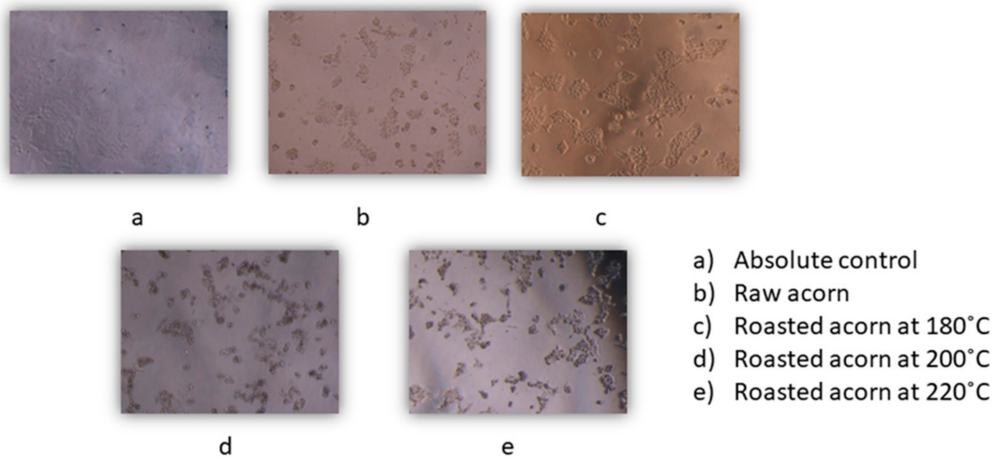

Figure 2. Microscopic aspects regarding the influence of aqueous acorn extracts on DLD-1 cells.

Table 7. Cell viability of the HFL-1 line.

		Aqueous Acorn Extract			
	Absolute Control	**180 °C**	**200 °C**	**220 °C**	**Raw**
OD450	0.675	0.537	0.546	0.53	0.493
OD450	0.623	0.541	0.564	0.45	0.5
Arithmetic mean	0.649	0.539	0.555	0.49	0.4965
Viability	100%	83.05	85.52	75.50	76.50

Performing the ordering according to the values obtained, the second result, with a relatively small difference from the first, belongs to the aqueous extract obtained from raw acorns with a viability of 76.5%. These are followed by aqueous extracts obtained from heat-treated acorns at 180 and 200 °C, registering a cell viability of 83.05% and 85.52%, respectively, compared to the absolute control.

It is also noted that the action of aqueous extracts on cell viability for the HFL-1 cell line, in this case, did not register values as low as in the case of the DLD-1 cell line.

We can recall that anti-proliferative effects have been reported for acorns of other *Quercus* species and other cell lines. For crude ethyl alcohol extract of *Quercus brantii* L. acorn, anti-proliferative effects have been reported by analyzing Hela cell lines (cervical adenocarcinoma), AGS (human gastric carcinoma) and HDFs (human dermal fibroblasts) [40], and anticancer effects have been reported (cell lines analyzed: HepG2 and THP-1) for *Quercus floribunda* L. [41]. While it is known that natural sources are of paramount importance, summarizing 60% of the currently used anticancer agents (Samarghandian et al., 2010), and the in vitro results are promising, in vivo studies are necessary to ultimately confirm a biological effect.

3.5. Sensory Evaluation of Chocolate Samples with Roasted Acorn Powder

The results of sensory evaluation are presented in Figure 3.

The panelists showed a preference for chocolate with cocoa powder, compared to chocolate with acorn powder, but the difference in general acceptance was very small, being 8.85 and 7.98, respectively. P0 (cocoa powder chocolate sample) fell between the scores 8 "I like it very much" and 9 "I like it very much", and P1 (roasted acorn powder chocolate sample) between the scores 7 "I like it moderately" and 8 "I like it very much", being very close to the score 8.

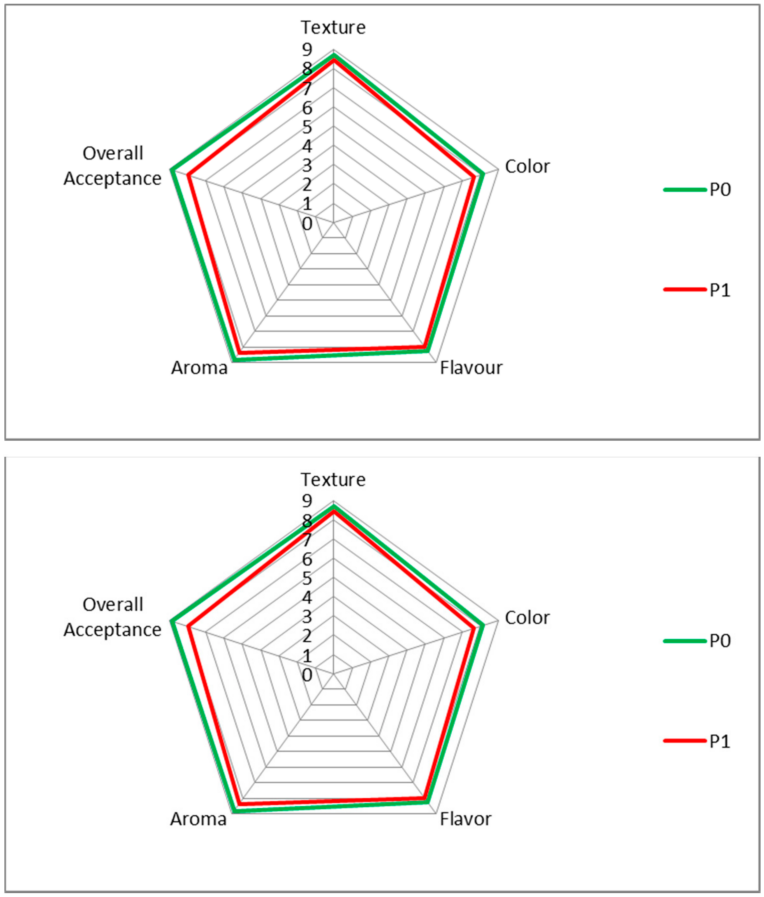

Figure 3. Sensory evaluation of chocolate with 7% cocoa powder (P0) and 7% roasted acorn powder (P1).

Regarding the other characteristics evaluated, acorn chocolate was liked by consumers between moderate and very much, falling between the scores 7.44 and 8.45, according to Figure 3. It should be noted that the texture has the highest score for both samples, indicating that the acorn powder addition did not influence this parameter.

Positive results regarding the appreciation of other acorn products have been reported for acorn bread [22,24,25], biscuits [26], muffins [27] or cake [28]. Moreover, some of them report a consumer preference for the acorn product, to the detriment of the classic product [24,25] of the same value [26].

4. Conclusions

This study presents an alternative to capitalize the red oak acorn powder to obtain a chocolate product that does not contain nervous system stimulants. Chocolate was obtained after optimizing the process of roasting acorns and obtaining the powder. Following the analyses performed, it was demonstrated that the red oak acorn is characterized by a high content of phenolic compounds responsible for numerous physiological, biological and biochemical functions due to their strong antioxidant activity. Red oak acorns are a promising base material with large amounts of secondary metabolites capable of providing protection against microbial contamination, especially having the ability to inhibit Gram-positive bacteria (*Bacillus cereus*). Following anti-proliferative tests, it was shown that aqueous acorn extracts, due to their antioxidant capacity and high polyphenol content, reduce the cell viability of HFL-1 and DLD-1 lines. Regarding the acceptability of acorn powder chocolate, following the results obtained, clear justifications can be provided regarding the applicability of acorn powder in the large-scale manufacturing of this type of product. In conclusion, this study showed that red oak (*Quercus rubra* L.) fruits are a potential alternative to cocoa powder in the manufacturing of chocolate.

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