Research Article **Open Access**

Transforming olive pits into functional foods: evaluation of phenolic, antioxidant, nutritional and microbiological properties

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Submission Date: September 18th, 2022; **Acceptance Date**: October 28th, 2022; **Publication Date**: November 7th, 2022

Please cite this article as: Galitsopoulou A., Salepi C., Karagianni F. Transforming olive pits into functional foods: evaluation of phenolic, antioxidant, nutritional and microbiological properties. *Functional Foods in Health and Disease* 2022; 12(11): 614-626. DOI: https://www.doi.org/10.31989/ffhd.v12i10.1011

ABSTRACT

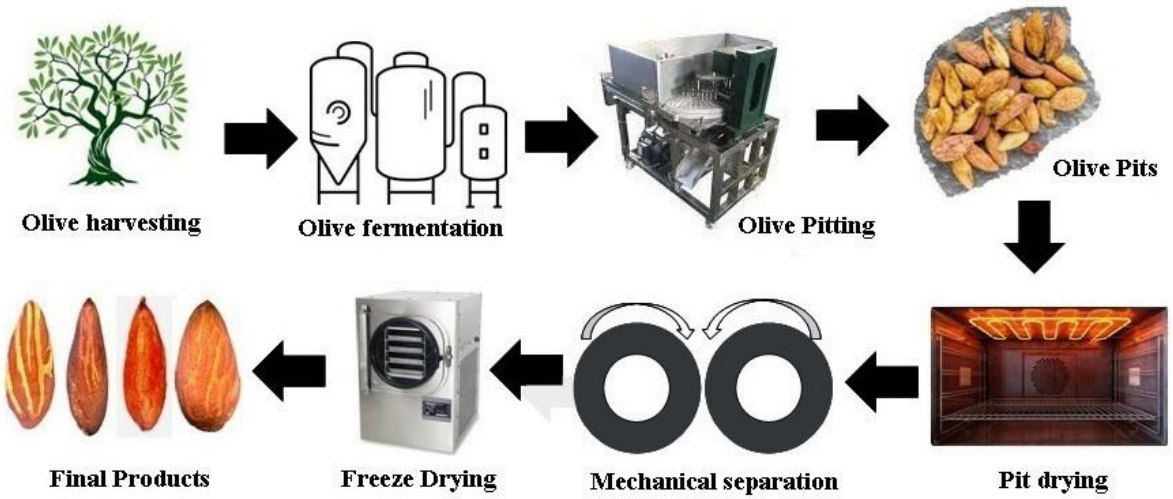
Background: The research aims to propose a new potential role for the olive pits as new functional foods for supporting human nutrition, health and wellbeing. The experimental research has been focused on developing a new method to suitably process the by-products of table olives (olive pits) and investigating the health-related and nutritional components of the final products.

Methods: A new methodology of processing the olive pit by-product to successfully resume the edible part of the pit interior, has been developed (as showed below): In the final form of the processed olive pit, a detailed identification and determination of specific phenolic compounds with pharmacological interest was developed with a high-performance liquid chromatography- photodiode array detector (HPLC-DAD). Antioxidant activity was also evaluated with DPPH free radical scavenging assay. Other health-related nutritional parameters were also investigated, with an emphasis on fatty acid profile analysis, dietary fiber and protein concentration. Microbiological quality of the final products were also investigated.

Results: Results showed that in the processed form of the olive pits, total bioactive phenolic content was found in significant levels, reaching an 8-25-fold higher concentration than the usual phenolic content of extra virgin olive oil. The quantitative determination showed that the principal biophenol determined was hydroxytyrosol, followed by tyrosol. DPPH analysis presented a high antioxidant activity, whilst the product presented considerable contents of monosaturated fatty acids, especially oleic acid, plant proteins and dietary fibers. Microbiological quality of the product was efficient in all samples tested.

Conclusions: After suitable processing, the by-products of the olive pits can be considered as a valuable source of bioactive phenolic compounds with strong antioxidant activity, as well as a good source of monosaturated fatty acids, oleic acid, plant proteins and dietary fibers. Overall, the olive pits could be reconsidered as a functional food or matrix with a promising potential for pharmaceutical, nutritional and cosmetic applications.

Keywords: olives; pits; phenols; antioxidants; monosaturated fatty acids; oleic acid; functional foods; by-products



INTRODUCTION

The Mediterranean region holds a leading position in the production of table olives. It is estimated that most of the world's production is produced in Spain, Italy, Greece, Portugal, France, Cyprus, Slovenia and Malta. Table olive processing contributes significantly to the economy of these countries, but also burdens them with a heavy environmental load. To date, the sustainable management of table olive waste is not systematically and effectively controlled due to many factors, such as large volume, seasonality, and complex waste composition. Among the different ways of waste treatments, sustainable utilization of by-products is considered a high priority because of its environmental and financial benefits [1]. For these reasons, experimental research has been focused on exploring new pathways of liquid and solid by-product utilization, with promising results concerning the recovery of health-relating substances, such as olive polyphenols.

Olive pits comprise the main solid by-product of the table olive industry, since table olives are mostly consumed as pitted products. The olive pits are usually removed during olive processing. Transforming olive pits into new forms of food could offer multiple benefits in the table olive industry, as well as in human nutrition. Therefore, this study is focused on investigating the methodology of processing the olive pits and further exploring their phenolic, antioxidant, nutritional and microbiological aspects, with the aim of transforming them from by-products to new forms foods, with nutritional, health and pharmacological benefits.

METHODS

Sampling: Four of the most important Greek table olive varieties (*Olea europea* sp.) Chalkidiki, Kalamata, Thassos and Konservolia were collected at their suitable maturation stage between 2019 and 2020. In total, 100 kg of olives were collected from each variety. After collection, the fresh olive samples were transferred to the laboratory and different industrial fermentation techniques were performed. For Chalkidiki, Kalamata and Konservolia olives, the Greek style processing with brine was selected. Chalkidiki variety was also processed with the Spanish-style technique. Thassos variety was treated with dry salting. Processing techniques were selected accordingly to industrial processing, as well as national legislation guidelines, since Spanish style lye-processing is not permitted in Kalamata, Konservolia or Thassos olives.

By-product olive pit processing: After fermentation was completed, the olives were mechanically pitted and the pits were kept for further processing. The pits were transferred to a laboratory thermal oven for 48 hours in 50^0C (model UN 55, Memmert GmbH + Co. KG, Germany). Afterwards, the pits were mechanically hulled with a custom-made pilot equipment, which consisted of two identical cylinders coated by polymeric foam. With this mechanism, the edible part of the pit was received in its intact spore form. Immediately after hulling, the olive spores were packaged in modified atmosphere (70% CO_2 / 30% N_2) high barrier bags and the appropriate methodology was investigated to reduce moisture content below 10% with freeze-drying (model Alpha 1-2LD, Christ, Germany).

Nutritional analysis: The research components of the nutritional analysis that were examined were a) protein content, b) total lipid content, c) dietary fiber content and d) fatty acid profile. The analytical methods applied for each parameter where: a) protein determination with AOAC Official Method 991.20, b) total lipid content with acid Hydrolysis – Soxhlet extraction c) dietary fiber determination with AOAC Official Method 985.29 and d) fatty acid profile as referred in the European Regulation No 2568/91.

Total phenolic content and identification of bioactive phenolic compounds: Plant extracts usually contain a combination of various types of bioactive compounds. The total content of the polyphenols in plants can be determined by the colorimetric method using the Folin-Ciocalteu reagent [2]. Furthermore, a more detailed identification and determination of specific phenolic compounds with pharmacological interest has been developed with a high-performance liquid chromatography- photodiode array detector (HPLC-DAD), in accordance with the International Olive Council COI/T.20/Doc No 29. Prior to analysis, a sample extraction preparation was performed as follows: the sample was first homogenized in a laboratory blender (T10 ULTRA-TURRAX®, IKA®-Werke GmbH & Co. KG, Staufen Germany) and then a quantity of the homogenized initial material was extracted with the ultrasound technique, using methanol as an extraction solvent, in a plant material/volume ratio solvent: 1/10, for a period of 30 minutes. Following the extraction, the sample was filtered on paper (Whatman® qualitative filter paper, Grade 1) and the filtrate was concentrated in a pre-weighed spherical flask at 40°C under vacuum. Using the technique of high-performance liquid chromatography with a photodiode array detector (HPLC-DAD) set at 280 nm, the following were performed: a) a qualitative analysis of phenolic components and b) an investigation of the detection and determination of specific biophenols that show

pharmacological interest and are considered in the health claims proposed for olives from the European Food Safety Authority.

Antioxidant activity: The antioxidant activity of the final products was estimated by the DPPH radical assay. The method was applied according to Spanou et al., 2008 [3] by using TROLOX as a standard (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid, TCI Chemicals, Portland, U.S.A.). 1,1-diphenyl-2-picrhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, U.S.A.). The other chemicals and solvents used were of the highest quality commercially available. Methanol of HPLC grade was used the primary solvent (Sigma-Aldrich, St. Louis, U.S.A.) Prior to the DPPH radical assay, the samples were suitably prepared by extraction based on a method developed for olive leaf products [4]. The results are expressed as mmol Trolox-equivalents per g of sample.

Microbiological analysis: Aerobic colony count determination was chosen as an indicator that can provide information about the general quality and shelf life and thus highlight potential problems of storage and handling. The method that was used was ISO 4833-1:2013.

Statistical evaluation: All samples were analyzed at least in triplicate. Results are reported as mean and standard deviation. A one-way analysis of variance (ANOVA) and Tukey's test were used to determine the significance of differences among the mean values at the 0.05 significance level; the analyses were carried out by IBM® SPSS® (Chicago, USA).

Analytical Quality Assurance: For the estimation of the analytical method performance, the following analytical criteria were calculated. Precision was estimated with the HORRAT value and measured to be less than 2. LOD, LOQ, reproducibility and recovery of the antioxidant and phenolic analysis method were estimated by the Trolox standard, as well as with certified reference substances (Sigma-Aldrich, St. Louis U.S.A.). The mean recovery of analytes ranged between 87% and 104%.

RESULTS AND DISCUSSION

Nutritional Composition: The results for the nutritional composition of the final products from the olive pits are presented in Table 1. The major nutritional component that was assessed is the lipid content, which varied from 39.7 to 55.8 g per 100g of sample. The experiment proceeded in a more detailed fatty acid profile analysis, demonstrating that monosaturated fatty acids were the major lipid acid group. According to experimental results, monosaturated fatty acids have been found between 26.81 to 42.44 per 100g of sample. The lipid profile investigation has also revealed that oleic acid is the major monosaturated fatty acid, with an average total percentage of 70% of the total monosaturated fatty acid concentration. Oleic acid was found in a significant concentration, ranging between 26.09-40.35 g $100g^{-1}$, depending on olive variety. Oleic acid is an important dietary and bioactive component, since a daily consumption of about 1½ tablespoons (20 grams) of oils containing at least 70% of oleic acid, may reduce the risk of coronary heart disease when replaced for fats and oils higher in saturated fat [5]. These results are in accordance with previous studies in the lipid profile of the edible part of the olives (flesh) and are considered to be important, due to the high significance of monosaturated fatty acids in human health [6].

Another interesting finding of this study is the high concentration of dietary fibers. The results showed that the dietary fiber content was estimated between 11.9-17.3 g per 100g of sample. Current results illustrate that

the samples of all olive varieties can be considered as a valuable source of dietary fibers, since they contain more than 6 g of dietary fiber per 100 g, which is the limit for a food high in dietary fiber according to the European legislation 1924/2004.

In regard to the protein content of the samples, results showed considerable levels between 8.9-18.1 g per 100 g. Whereas the protein concentration of the samples is lower than the previously noted dietary components, it is also considered to be of high importance, since the average value is estimated to be more than 12% in terms of energy, as stated in European legislation (EC) 1924 (2006) for nutritional claims. Therefore, the products can be considered a good source of proteins. These results are of an emerging significance since more people are becoming interested in following vegan diets or reducing their use of animal products. This shift is increasing the need for new sources of plant proteins, in the form of functional foods (FFs), as products that might help prevent disease progression or optimize health, thereby reducing healthcare costs and improving the quality of life for many [7].

Table 1. Nutritional composition (mean and standard deviation values) and microbiological quality of the olive by-products.

Parameter (g/100g)	Chalikidiki sp.	Kalamata	Thassos	Amfissa	Chalkidiki nat.*
Fat, Total	48.6±6.1^{a}	46.3±5.7^{a}	39.7±4.6^{b}	55.8±7.4^{c}	42.6±5.2^{a}
M. Fatty Acids	34.59±4.3^{a}	32.74±3.9^{a}	26.81±3.4^{b}	41.41±4.4^{c}	42.44±4.6^{c}
Protein	9.3±31.1^{a}	10±1.3^{a}	18.1±1.3^{b}	12.2±1.8^{c}	8.9±1.4^{a}
Oleic Acid	33.79±3.4^{a}	31.77±3.2^{a}	26.09±2.3^{b}	40.35±4.3^{c}	33.38±3.3^{a}
Dietary Fiber	13.3±2.2^{a}	16.4±2.7^{b}	13.8±1.9^{a}	17.3±2.5^{b}	11.9±2.2^{a}
aw	0.77^{a}	0.79^{a}	0.53^{b}	0.72^{a}	0.75^{a}
Aerobic Plate Count (cfu/g)	<10	<40	<10	<40	<10

*Chalkidiki NAT. is Chalkidiki variety with natural (Greek style) fermentation. whereas Chalkidiki SP. has been processed with lye (Spanish-style processing). **Different letters stand for statistically significant differences at $p < 0.05$.

A statistical evaluation between the differences in nutrient contents from different varieties was also tested. Results showed significant differences among most of the nutrients ($P<0.05$, presented in table 1). Amfissa by-products showed the highest concentration in lipid and dietary content, whereas Chalkidiki variety with natural fermentation presented the highest levels in monosaturated fatty acid. Interestingly, the salt content remained in the same levels in all varieties tested ($P>0.05$), which can be attributed to the similarities of the processing methods applied.

Total phenolic content: The olive pit by-products have been evaluated for their total phenolic content with the Folin – Ciocalteau method and the results are presented in Figure 1. The total phenolic concentration varied from 5692 to 13030 mg kg^{-1}, with an average of 8073 mg kg^{-1}. These values are 40-fold higher than the 200 mg/kg amount stated by EFSA related to health claims for polyphenols in olives, meaning that 0.5 g of the product can cover the same content of polyphenols as 20 g of olive oil.

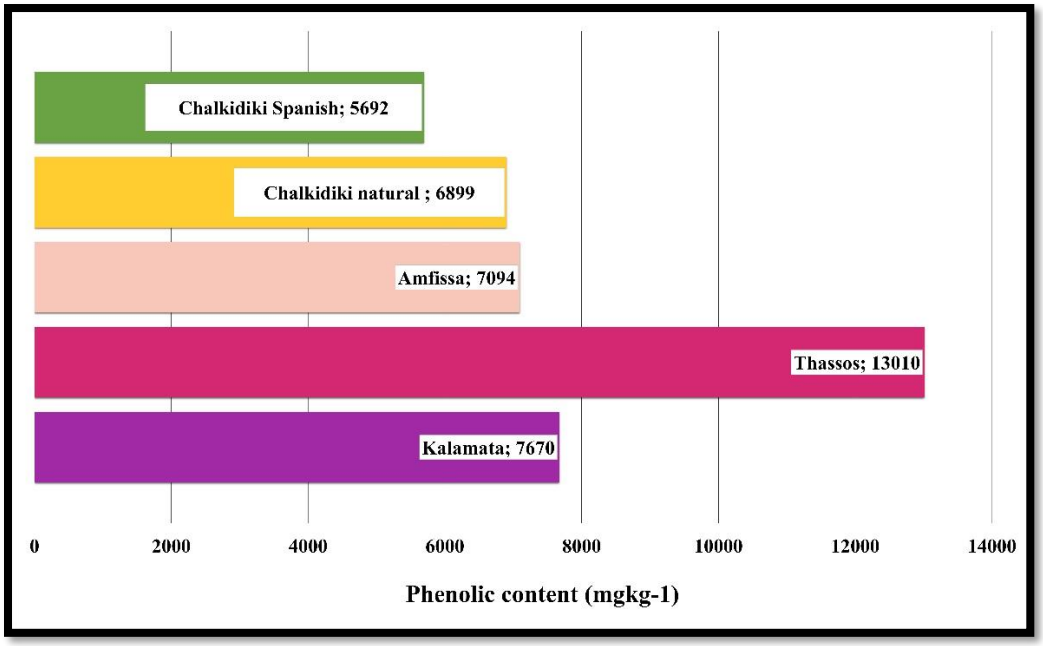


Figure 1. Total phenolic content of olive pit by-products from different olive varieties. Results are expressed as mg of Tyrosol per kg of product.

A more detailed statistical evaluation among olive varieties showed that Thassos variety presented the highest concentration in phenolic compounds, Kalamata variety had the second highest levels, whilst the Chalkidiki variety with NaOH processing presented the lowest values. The difference in phenolic content among varieties was significantly different ($P<0.05$) and can be attributed to various factors, such as generic, agronomic and processing conditions.

The results of this study also illustrate that Thassos olives contain almost twice the phenolic compounds compared to the other varieties tested. This result is in accordance with previous results, which presented up to 4792 total phenolic compounds (μM) for this cultivar [8]. Thassos olives are neither processed with NaOH or NaCl solution. It is well known that olive processing, especially the debittering stage can affect the phenolic content of the final products, so the Thassos variety has the highest phenolic concentration due to a less invasive processing technique. Kalamata, Amfissa and Chalkidiki natural fermentation olives also presented high phenolic contents, attributed to the Greek-style natural fermentation method that does not involve chemical debittering. On the contrary, Chalkidiki with Spanish-style NaOH processing presented the lowest phenolic content, a process that can significantly reduce phenolic concentration in table olives [9].

A comparative study of previous literature showed that the samples contained an average of 50-200% more phenolic compounds than the olive flesh, depending on olive variety and processing conditions [10, 11, 12]. This can be attributed to metabolic and physiological factors that lead to a higher concentration of phenolic compounds inside the pit, in comparison to the olive flesh. Moreover, it is also possible that the woody pit hull acts as a protective barrier to nutritive compounds inside the pit. The results of this study also presented considerably higher levels of phenols, in comparison to extra virgin olive oil (EVOO). In fact, the level of phenolic compounds in extra virgin olive was reported to be between 50 and 1000 mg/Kg, with usual values between 100 and 300 mg/kg, depending on the variety, harvest time, production technique and the shelf-life condition of the final product [13,14]. Phenolic content could be also affected by geographical origin, as it has been noted for other Mediterranean products [15]. The results of the

current study show that the average content of 8073 mg kg^{-1} is 8-25-fold higher than the EVOOH phenolic content of previous reported values.

Identification of bioactive phenolic compounds: EFSA scientific opinion on the substantiation of health claims related to polyphenols in olives specifies that the food constituent that is the subject of the health claims is hydroxytyrosol and oleuropein complex (EFSA Journal 2011;9(4):2033). The conditions of use specify 2-15 mg per day of hydroxytyrosol or oleuropein complex for the following effects: may help maintain healthy LDL cholesterol level and lipid oxidation/antioxidants ID 1638, maintenance of normal blood HDL-cholesterol concentrations 1639, antioxidant activity and the protection of body cells and LDL from oxidative damages ID 1696. Under these guidelines, the current study performed a detailed analysis and identification of specific bioactive phenolic compounds, showing strong pharmacological interest with ion-pair reversed phase-high performance liquid chromatography coupled to a diode array detector RP-HPLC (DAD).In total, 12 biophenol compounds were investigated: [1] Hydroxytyrosol, [2] Tyrosol, [3] Syringic acid, [4] Oleacein, [5] Oleuropein, [6] Oleocanthal, [7] Pinoresinol, [8] 1-acetoxy-pinoresinol, [9] Oleuropein aglycon, [10] Luteolin, [11] Apigenin, [12] Ligstroside aglycon. The chromatography results are presented in figure 2.

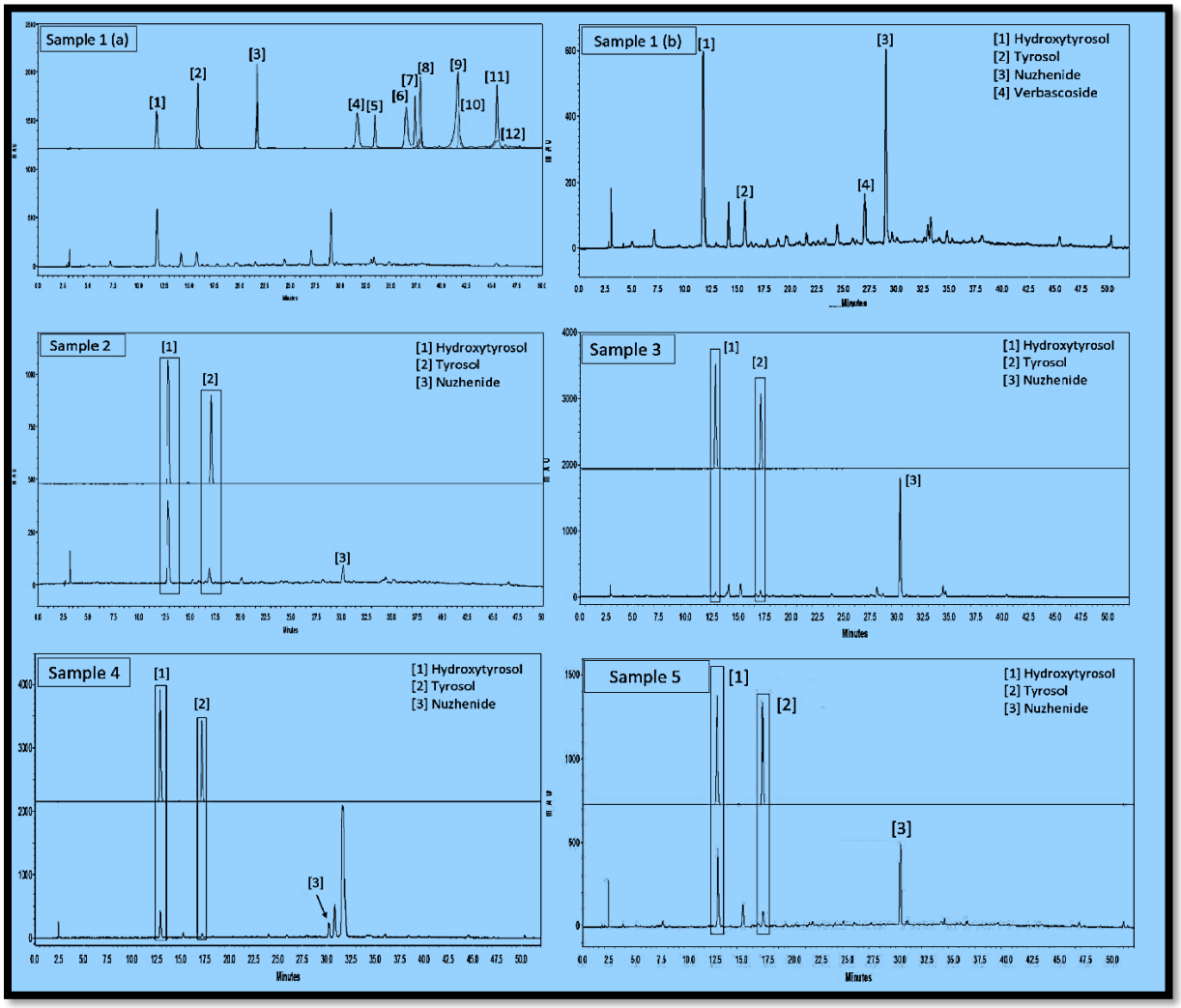


Figure 2. HPLC-DAD determination of biophenols. Sample 1 (a): The blue, red, green lines correspond to the biophenol standards used: [1] Hydroxytyrosol, [2] Tyrosol, [3] Syringic acid, [4] Oleacein, [5] Oleuropein, [6] Oleocanthal, [7] Pinoresinol, [8] 1-acetoxy-pinoresinol, [9] Oleuropein aglycon, [10] Luteolin, [11] Apigenin, [12] Ligstroside aglycon. Sample 1 (b): Kalamata. Sample 2: Chalkidiki natural. Sample 3: Thassos. Sample 4: Chalkidiki Spanish-style 5: Amfissa.

During RP-HPLC determination, an interesting bioactive component was detected and identified as nuzhenide. Although it was not mentioned in the primary list of biophenols, nuzhenide is a bioactive secoiridoid and with the monoaldehydic form of oleuropein aglycon, it presents a strong pharmacological interest with important antioxidant properties [16, 17,18].

The analysis of all varieties showed that the principal bioactive phenol determined was hydroxytyrosol, followed by tyrosol. Hydroxytyrosol and tyrosol are among the main biologically active components of the olive that have been attributed to valuable biological actions, including antioxidant, antimetastatic, antimicrobial, antiviral and antiaging. They simultaneously present neuroprotective and other nutraceutical activities both in *vitro* and *in vivo* studies [19, 20, 21, 22, 23]. In regard to the rest of the bioactive phenols investigated, it appears that the levels of oleuropein were < 40 mg, Oleasein < 10 mg, and Oleocanthal < 10 mg per 100 g of samples.

A detailed quantification of the primary biophenols hydroxytyrosol and tyrosol is presented in Table 2. Hydroxytyrosol levels varied between 85.82-140.78 mg per 100g, with an average of 109.28 mg per 100g. Tyrosol values varied between 22.32 - 59.82 mg $100g^{-1}$, with an average of 36 mg $100g^{-1}$. Altogether, both biophenols reached the total of 200 mg $100g^{-1}$, with an average of 144.13 mg $100g^{-1}$. These values are considered higher than the usual values for table olives [24] and olive oil [25], although phenolic composition in olives is complex and depends upon many factors, such as variety and maturation stage. The Panel of EFSA considers that polyphenols standardized by their content of hydroxytyrosol and its derivatives (e.g., oleuropein complex) are sufficiently characterized in relation to the claimed effects, with a proposed daily dose of 2-5 mg. Under this scheme, a quantity of 2-4 g of the final product of olive pits is sufficient to cover these needs.

Table 2. Quantification of Hydroxytyrosol and Tyrosol in the analyzed samples from different olive varieties. Results in mg per 100g of sample.

Parameter	Chalikidiki SP.	Kalamata	Thassos	Amfissa	Chalkidiki nat.*
Hydroxytyrosol	85.82	140.78	120.73	92.45	106.63
Tyrosol	22.32	59.82	28.39	31.76	37.96
Sum	108.14^{a**}	200.6^{b}	149.12^{c}	124.21^{d}	138.59^{d}

*Chalkidiki NAT. is Chalkidiki variety with natural (Greek style) fermentation. whereas Chalkidiki SP. has been processed with lye (Spanish-style processing). **Different letters stand for statistically significant differences at $p < 0.05$.

Antioxidant activity: Antioxidants are the compounds which counteract free radicals by intervening at any one of the three major steps of the free radical mediated oxidative process. The analytical method of α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging has been applied in the by-products of the olive pits, as well as after drying and freeze drying. The results are presented in Figure 3.

All olive varieties presented significant antioxidant activity, which varied from 36.1-84.4 mmole/g. Thassos variety exhibited the major antioxidant activity, whilst Chalkidiki with Spanish fermentation presented the lowest levels. Drying of the samples led to a reduction in their antioxidant activity. The percentage of reduction was higher with heat-drying of the samples. On average, heat drying resulted in a 30% loss of antioxidant activity.

On the contrary, freeze-drying preserved 90% of the antioxidant activity of the fresh samples; Thus, it is considered better for reducing moisture content, to improve their shelf life and preservation stability.

In regard to antioxidant levels in previous studies for similar products, the values of the current assay are considerably higher compared to previous studies for olives [26], olive leaves [27], olive oil [28, 29, 30] and olive oil mill waste waters [31]. The current assay demonstrates that pit olive by-products present substantial antioxidant activity and they could be reconsidered as a good source of natural antioxidants, with possible applications in the pharmaceutical and food industries as in previous studies 32, 33]. However, more thorough steps are necessary to scale-up these products and bring them to a final market form as functional foods (FFs). The 16-step process proposed by the Functional Food Center (FFC) will be beneficial in establishing the benefits, as well as the marketability of these products in the international market [34].

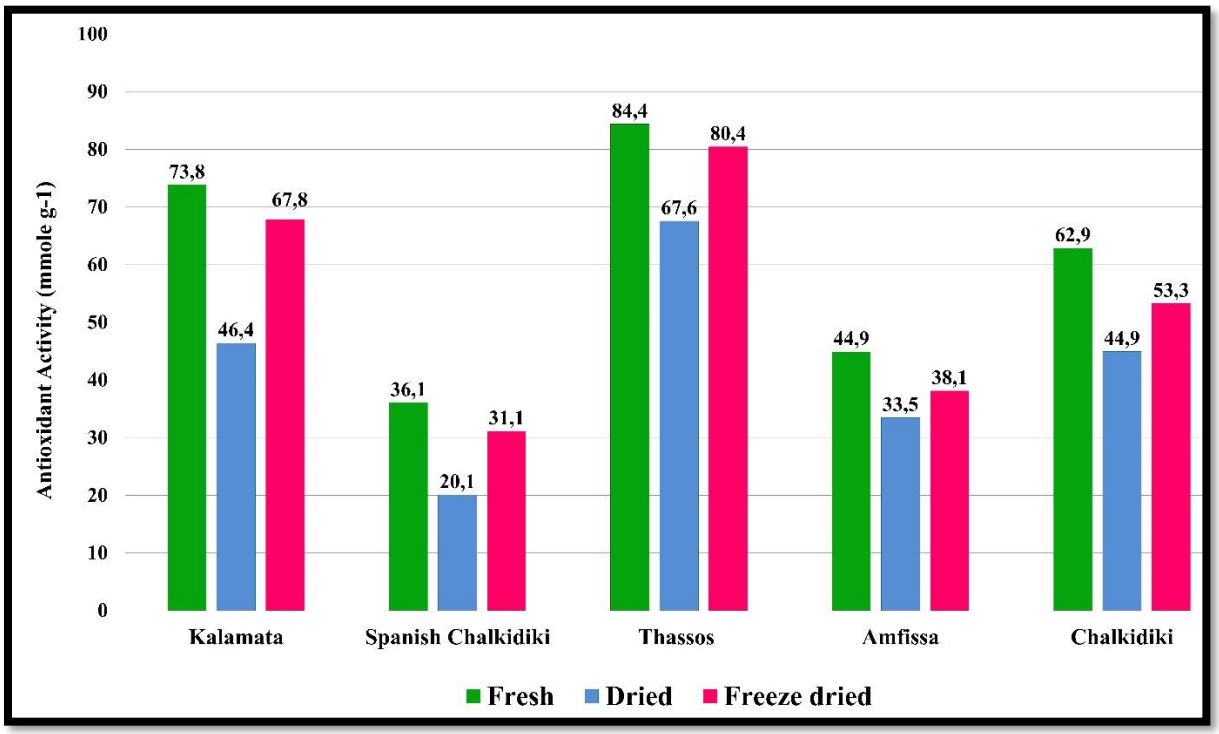


Figure 3. Antioxidant activity of the by-products of the olive pits, in three forms a) fresh, b) dried c) freeze-dried in Kalamata, Chalkidiki Spanish processing, Thassos, Amfissa and Chalkidiki natural olives. Results in mmol Trolox-equivalents per g of sample.

Microbiological quality: In order to determine the microbiological quality of the final products, a microbiological analysis of aerobic plate count (ACC) was performed, and the results are presented in Table 1. The results presented an excellent microbiological quality of all the samples examined. It is interesting to note that most of the samples presented a very low enumeration of microorganisms, in terms of <10 cfu/g and only two varieties presented a value of <40 cfu/g. These results can be attributed to two factors: one is the low water activity of the final samples, which is on average 0.73 (showed in table 1), a value that can reduce the activity of most microorganisms. However, by-products probably contain polyphenols that can act as antimicrobiological agents.

The microbiological assay did not expand in specific pathogens, since the ACC levels were in low levels. Regarding the statistical evaluation among varieties, only Thassos variety showed significantly lower water activity than other varieties ($P<0.05$), which is attributed to the dry salting processing, as opposed to the liquid brine processing for the remainder of the samples. Nevertheless, there are no previous studies concerning olive pit products and microbiological quality, so it is difficult to compare these results with previous findings.

CONCLUSION

The present study encompassed a new methodology of processing the olive pits of popular Greek olive varieties, after industrial style fermentation and further processing. The proposed experimental method that was developed included hulling of the external shell, mechanical separation of the interior, preservation in modified atmosphere and reduction of the excess moisture of the edible part of the olive pits by heating or freeze-drying. Afterwards, experimentation of the nutritional, pharmacological and microbiological aspects of the final products were performed, with an emphasis on the polyphenol, as well as the antioxidant aspects that are related to human health and well-being.

Experimental results of this study showed that olive pit by-products in their final form present an excellent microbiological quality and also contain significant amounts of several bioactive and nutrient components, such as monosaturated fatty acids, dietary fibers, plant proteins and oleic acid. A particular focus was given in the phenolic profile and quantification of bioactive phenols associated with health-related benefits. Total phenolic content was found interestingly high, as it was estimated 8-25-fold higher than the usual EVOOH phenolic content. Hydroxytyrosol and tyrosol were identified as the primary active biophenols and quantification analysis showed that 2-4g of the final product can cover the biophenol daily needs, as stated by the European Food Safety Authority.

In addition, DPPH analysis presented a high antioxidant activity of all olive varieties. Thus, confirming that final products are a valuable source of natural antioxidants with a good potential for pharmaceutical, functional food and cosmetic uses. Therefore, the proposed methodology will be suitable to be tested in an upscaled industrial environment, to improve the sustainability performance of the olive industry, as well as to offer new functional foods with multiple nutritive and health-related active components. Future research projects may be useful to focus on optimizing the industrial scale-up processing of the table olive pits, as well as thoroughly investigating the benefits, as well as the marketability of these products, with a methodology as proposed by the Functional Food Center (FFC).

Abbreviations: HPLC-DAD: high performance liquid chromatography- photodiode array detector, DPPH: 2,2-diphenyl-1-picrylhydrazyl, AOAC: Association of Official Agricultural Chemists, TROLOX: 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid, ANOVA: analysis of variance, LOD: limit of detection, LOQ: limit of quantification, EFSA: European Food Safety Authority, EVOOH: extra virgin olive oil, FFs: functional foods, RP-HPLC: reverse phase- high performance liquid chromatography

Author's Contributions: Dr. Agostina Galitsopoulou: designed the research, performed statistical analyses and wrote the manuscript. Chrisa Salepi: provided sample processing and supported the research. Foteini Karagianii conducted the research. Dr. Agostina Galitsopoulou: had primary responsibility for the final content. All authors read and approved the final version of the manuscript.

Competing Interests: There are no conflicts of interest to declare.

Acknowledgements and Funding: This research has been co-financed by the European Regional Development Fund of the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH – CREATE – INNOVATE (project code: T2EDK-00284

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