

Article

Olive Leaf Powder as a Potential Functional Component of Food Innovation: An In Vitro Study Evaluating Its Total Antioxidant Capacity and Phenolic Content

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Abstract

Olive leaves (*Olea europaea*) are the most abundant agricultural by-product of olive tree cultivation, generating substantial waste each year. Their disposal is deemed an environmental challenge, particularly in Mediterranean countries that dominate the olive oil sector, yet their rich bioactive profile makes them promising candidates for functional food development. This study aimed to determine the total antioxidant capacity (TAC) and total phenolic content (TPC) of olive leaf powder extracts using different extraction solvents and methods to identify the most efficient strategy for possible incorporation into functional food systems. Extractions were performed with distilled water, 70% ethanol, 80% methanol, and 50% acetone using three methods: stirring, soaking, and ultrasound-assisted extraction (UAE). TAC and TPC were quantified using the FRAP and Folin–Ciocalteu assays, respectively. Among solvents, acetone consistently yielded the highest values across most methods (TAC: 19.02 mmol Fe²⁺/L, TPC: 1289.95 mg GA/L), while ethanol also showed strong extraction performance (TAC: 15.35 mmol Fe²⁺/L; TPC: 1214.76 mg GA/L), offering a safer and more scalable option for food applications. Method-wise, UAE achieved the greatest phenolic recovery, while both UAE and stirring proved effective for antioxidant extraction. Overall, these findings provide quantitative evidence supporting possible incorporation of olive leaf powder as a valuable ingredient in functional foods and other sustainable applications, while also contributing to the circular economy through the sustainable valorization of agricultural waste.

Keywords: functional foods; olive leaf powder; total antioxidant capacity; total phenolic content; ultrasound-assisted extraction; natural antioxidants; organic solvents; FRAP assay; Folin–Ciocalteu method



Academic Editor: Monika Gibis

Received: 14 July 2025

Revised: 8 August 2025

Accepted: 20 August 2025

Published: 28 August 2025

Citation: Almpounioti, K.; Papagianni, O.; Michaloudi, P.; Tsermoula, S.K.; Potsaki, P.; Dimou, C.; Koutelidakis, A.E. Olive Leaf Powder as a Potential Functional Component of Food Innovation: An In Vitro Study Evaluating Its Total Antioxidant Capacity and Phenolic Content. *Appl. Sci.* **2025**, *15*, 9462. <https://doi.org/10.3390/app15179462>

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1. Introduction

Functional foods have steadily gained significant market traction in recent decades mainly due to increased consumer awareness regarding the role of diet in disease prevention, overall health, and environmental consciousness [1,2]. Recent analyses support this trend by observing a gradual shift in consumer preferences towards healthier, more sustainable food options that are enriched with bioactive compounds, which, in turn, has prompted the food industry to explore innovative applications and recipes [1,3,4]. As a result, there is a continuous search for natural, safe, and cost-effective sources of bioactive

compounds that can be integrated as ingredients into food systems while at the same time supporting circular economy principles [5].

Olive leaves (*Olea europaea*) are the largest by-product of olive tree cultivation. Although they have been increasingly investigated in recent years for use in applications ranging from dietary supplements and beverages to medicinal and cosmetic uses, their large-scale valorization in the food industry remains limited [5,6]. Their rich bioactive profile has been well documented, specifically due to their high concentration in polyphenols such as oleuropein and hydroxytyrosol, all of which exhibit significant antioxidant, anti-inflammatory, antihypertensive, and antimicrobial properties [7,8]. These characteristics make olive leaves a promising ingredient for inclusion in functional food formulations.

Several recent studies have evaluated different extraction techniques and solvent systems for recovering phenolic compounds from plant materials such as olive leaves [5,6]. However, there is currently a lack of standardized methods that would allow for consistent characterization of their composition and nutritional profile, since findings often vary depending on the matrix, target compounds, and methodological parameters, making it difficult to draw direct comparisons or determine a universally superior extraction strategy [9]. This therefore highlights the importance of comparative evaluations and analysis under controlled conditions in order to better understand how solvent choice and extraction method influence bioactive recovery.

Organic solvents such as methanol, ethanol, and acetone have been widely used in polyphenol research due to their strong extraction capabilities [5]. Ethanol, in particular, is especially relevant for food-related applications given its recognized safety status (GRAS), while methanol and acetone, though not as suitable for use directly in food products, are valuable in optimization studies [10,11]. Water, although less efficient in terms of extraction yield, is directly compatible with food systems and presents no toxicological concerns. In the present study, we used aqueous ethanol 70 vol%, aqueous methanol 80 vol%, and aqueous acetone 50 vol% as our organic solvents, utilizing them in concentrations frequently cited in the literature as most effective for polyphenol recovery from plant materials.

The goal of this study was to evaluate the performance of three extraction methods (stirring, soaking, and ultrasound-assisted extraction) in recovering total antioxidant capacity and total phenolic content from olive leaf powder using different solvent systems. By conducting comparisons between the selected solvent systems, analyzed for each extraction method, our study aims to provide practical knowledge for optimizing olive leaf extraction, thus promoting sustainable valorization of this abundant agricultural by-product within the framework of circular economy principles.

2. Materials and Methods

2.1. Sample Preparation

Fresh leaves from olive trees—the Koroneiki variety (*Olea europea* var. *macrocarpa* alba)—were purchased from A.S. Dolon (agricultural cooperative, Kalamata, Greece). The leaves, derived from three tree parts, were harvested in October 2024 and mixed in equal proportions to ensure biological diversity and sample homogeneity. After manual removal of foreign material, the leaves were washed with deionized water and dried with absorbent paper. They were then cut into 5 mm pieces and dried in a laboratory oven at 70 °C for 150 min. These drying conditions were selected following a preliminary trial, where different drying conditions were tested by monitoring leaf weight at multiple time points and temperatures. The selected conditions (70 °C, 150 min) were the mildest effective treatment that reduced the moisture content without further weight loss, thus minimizing thermal degradation while ensuring dehydration. The dried leaves were ground into a fine

powder (20–30 mesh) using a laboratory grinder (IKA M 20, IKA, Staufen, Germany) and stored in centrifuge tubes at 4 °C until further analysis.

2.2. Solvent Preparation

Absolut methanol, ethanol, and acetone were purchased from Sigma Aldrich Co. LLC (Munich, Germany). Deionized water (dH₂O), aqueous ethanol 70 vol%, aqueous methanol 80 vol%, and aqueous acetone 50 vol% were used as extraction solvents. Distilled water was used to prepare the solutions of ethanol/water 70:30 (*v/v*), methanol/water 80:20 (*v/v*), and acetone/water 50:50 (*v/v*), while also being used as a single aqueous solvent.

The selected solvent-to-water ratios (70% ethanol, 80% methanol, and 50% acetone) were based on prior studies in the literature reporting enhanced extraction efficiency of phenolic and antioxidant compounds at these concentrations.

2.3. Extraction Procedures

For each solvent, three extraction methods were employed, with these methods being applied in triplicate:

2.3.1. Continuous Stirring Extraction

Samples of 10 g of olive leaf powder were mixed with 100 mL of each solvent in 250 mL Erlenmeyer flasks (Merck KGaA, Darmstadt, Germany). Samples were continuously agitated in a shaking incubator (Giorgio Bormac Srl., Carpi, MO, Italy) at 25 °C and 250 rpm for 1 h. The extracts were then filtered using Whattman filter paper (Thermo Fisher Scientific, Waltham, MA, USA) and then centrifuged using a benchtop refrigerated centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at 10,000 rpm. Their supernatant fluids were collected for further analysis.

2.3.2. Soaking Extraction

The same proportion of olive leaf powder samples (10 g) were combined with 100 mL of each solvent in four 250 mL Erlenmeyer flasks (Merck KGaA, Darmstadt, Germany) and left undisturbed in the dark at 25 °C for 4 h. The extracts were then filtered and centrifuged using a benchtop refrigerated centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) at 10,000 rpm for 10 min. The supernatants were collected for analysis.

2.3.3. Ultrasound-Assisted Extraction

Samples of 10 g of olive leaf powder and 100 mL of solvent were added to 200 mL Erlenmeyer flasks (Merck KGaA, Darmstadt, Germany) and were subjected to ultrasonic treatment in an ultrasonic water bath at 60 °C for 10 min (frequency, 35 kHz; power, 60/120 W; Argolab Via della Meccanica, Carpi, MO, Italy). The extracts were filtered and centrifuged at 10,000 rpm for 10 min using a benchtop refrigerated centrifuge (Thermo Fisher Scientific, Waltham, MA, USA), before the supernatants were collected for analysis.

2.4. Sample Dilutions

All supernatants were diluted with deionized water (dH₂O) at levels of 1:5 (*v/v*), 1:10 (*v/v*), and 1:20 (*v/v*), ensuring absorbance values remained within the linear range (0.1–1.0 AU) of the Lambert–Beer law.

2.5. Analytical Methods

2.5.1. Total Antioxidant Capacity (FRAP Assay)

The total antioxidant capacity (TAC) was evaluated for all samples by performing the Ferric Reducing Antioxidant Power assay (FRAP assay). Specifically, FRAP reagent was prepared by mixing 25 mL acetate buffer (0.4 M, pH 3.6), 2.5 mL TPTZ solution, and

2.5 mL FeCl_3 solution. In a 96-well plate, 80 μL of FRAP reagent and 20 μL of sample (in triplicate) were added to each well and then incubated in darkness for 30 min (room temperature). The absorbance was measured with a Spark multimode plate reader (Tecan U.S. Inc., Chapel Hill, NC, USA) at 595 nm.

2.5.2. Total Phenolic Content (Folin–Ciocalteu Assay)

The total phenolic content (TPC) of the tested samples was evaluated by performing the Folin–Ciocalteu assay. Briefly, in a 96-well plate (Merck KGaA, Darmstadt, Germany), 50 μL of each diluted sample were incubated in the dark for 30 min (room temperature), along with 20 μL of Folin–Ciocalteu reagent and 20 μL of sodium carbonate solution (7.5%). The absorbance was evaluated with a Spark multimode plate reader (Tecan U.S. Inc., Chapel Hill, NC, USA) at 765 nm.

2.6. Data Analysis

The TAC and TPC of olive leaf extracts were analyzed independently for each extraction method (stirring, soaking, ultrasound-assisted), as differences in experimental parameters (extraction time, concentrations) prohibited direct statistical comparisons between methods. Statistical significance was assessed only within each method to evaluate differences between solvents under their respective conditions.

All experiments were performed in triplicate. Data are expressed as mmol Fe^{2+} equivalents/L for TAC and $\text{mg Gallic Acid equivalents (GAE)/L}$ for TPC. To reflect analytical precision, data in tables are presented as mean $\pm$ standard deviation (SD). Statistical analysis was conducted for each method tested by performing one-way ANOVA tests, followed by Bonferroni's post hoc tests, to determine significant differences among means. The level of statistical significance was set at $p < 0.05$. Data analysis was performed using IBM SPSS Software v.21 (SPSS Inc., Chicago, IL, USA).

3. Results

In this study, the total antioxidant capacity (TAC) of olive leaf extracts was determined using the FRAP assay, and the total phenolic content (TPC) was measured using the Folin–Ciocalteu method.

3.1. Total Antioxidant Capacity (TAC)

The TAC of samples evaluated in each method are summarized in Table 1 and graphically presented in Figure 1.

Table 1. Total antioxidant capacity (TAC) of olive leaf extracts obtained using different extraction methods and solvents.

Extraction Method	Solvents			
	Water	80% Methanol	70% Ethanol	50% Acetone
Stirring	9.71 ± 0.88^d	12.12 ± 1.12^c	15.35 ± 0.71^b	19.01 ± 0.96^a
Soaking	12.29 ± 0.95^c	$14.63 \pm 0.57^{b,c}$	$14.77 \pm 3.90^{b,c}$	19.02 ± 1.43^a
Ultrasound	14.49 ± 0.67^b	15.80 ± 0.82^a	10.33 ± 0.56^c	16.51 ± 1.57^a

Values are presented as mean $\pm$ SD. TAC is expressed as mmol Fe^{2+} equivalents per liter of olive leaf extract. Different superscript letters within each row indicate significant differences ($p < 0.05$).

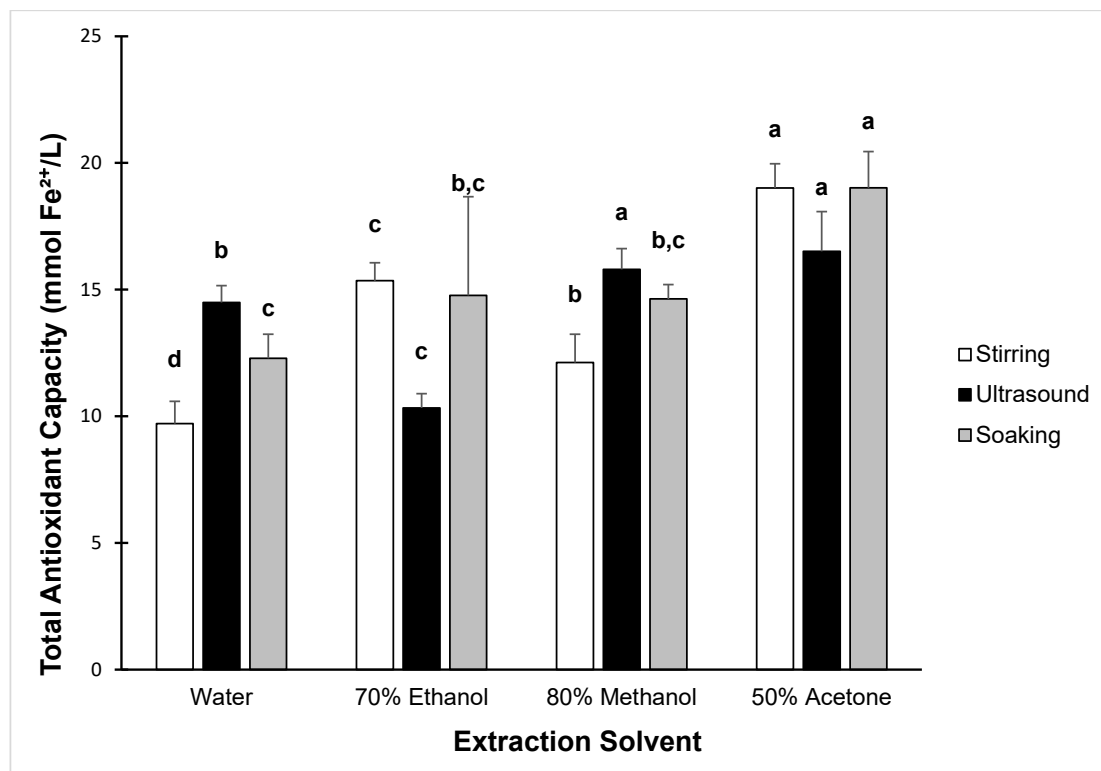


Figure 1. Total antioxidant capacity (TAC) of olive leaf powder extracts using four different solvents (aqueous ethanol 70 vol%, aqueous methanol 80 vol%, aqueous acetone 50 vol%, and distilled water) across three extraction methods: stirring, soaking, and ultrasound-assisted extraction (UAE). TAC was assessed using the FRAP assay and is expressed as mmol Fe²⁺ equivalents per liter of extract. Data is presented as mean $\pm$ standard deviations ($n = 3$). Different lowercase letters above bars indicate statistically significant differences ($p < 0.05$) between solvents within the same extraction method, as determined by Bonferroni's post hoc test. No statistical comparison was conducted between different extraction methods due to differing conditions.

3.1.1. Stirring Extraction

The data obtained following stirring extraction are graphically presented in Figure 1. The TAC of olive leaf extracts varied significantly, across the use of different solvents (Table 1, Figure 1). Aqueous acetone yielded the highest TAC (19.01 mmol Fe²⁺/L), followed by aqueous ethanol and aqueous methanol, while aqueous extracts exhibited the lowest values (9.71 mmol Fe²⁺/L). Statistical analysis and post hoc tests confirmed significant differences between all solvents, with acetone showing statistically superior performance ($p < 0.05$).

3.1.2. Soaking Extraction

Figure 1 presents the results obtained following the soaking extraction method. The prolonged soaking method slightly increased TAC in the sample, which was solved with aqueous acetone (19.02 mmol Fe²⁺/L), while it greatly improved TAC in the sample extracted with aqueous methanol (80%); however, its use resulted in a slight decrease in the TAC of the extract with aqueous methanol (Table 1). ANOVA tests confirmed acetone's superiority, while aqueous extracts remained the least effective 12.29 mmol Fe²⁺/L, ($p < 0.05$).

3.1.3. Ultrasound-Assisted Extraction

Ultrasound-assisted extraction enhanced TAC for the aquatic and methanolic extraction (Table 1). As presented in Figure 1, the extracts of aqueous methanol and aqueous acetone achieved the highest values with this method (15.80 ± 0.82 and 16.51 ± 1.57 mmol

Fe^{2+} /L respectively), while the sample extracted in aqueous ethanol underperformed (10.33 ± 0.56 mmol Fe^{2+} /L). Our ANOVA test revealed significant differences, with the aqueous ethanolic extracts being statistically distinct from aqueous methanolic and samples solved in aqueous acetone ($p < 0.05$).

3.2. Total Phenolic Content (TPC)

The TPC of samples assessed in each method are summarized in Table 2 and graphically presented in Figure 2.

Table 2. Total phenolic content (TPC) of olive leaf extracts obtained using different extraction methods and solvents.

Extraction Method	Solvents			
	Water	80% Methanol	70% Ethanol	50% Acetone
Stirring	616.19 ± 113.60^c	701.56 ± 127.60^c	815.72 ± 119.97^b	1152.70 ± 164.60^a
Soaking	665.54 ± 67.78^c	762.93 ± 92.38^b	1100.07 ± 151.10^a	1193.63 ± 145.34^a
Ultrasound	794.92 ± 90.33^b	914.08 ± 147.38^b	1214.76 ± 190.74^a	1289.95 ± 191.17^a

Values are presented as mean $\pm$ SD. TPC is expressed as mg gallic acid equivalents (GAE) per liter of extract. Different superscript letters within each row indicate significant differences ($p < 0.05$).

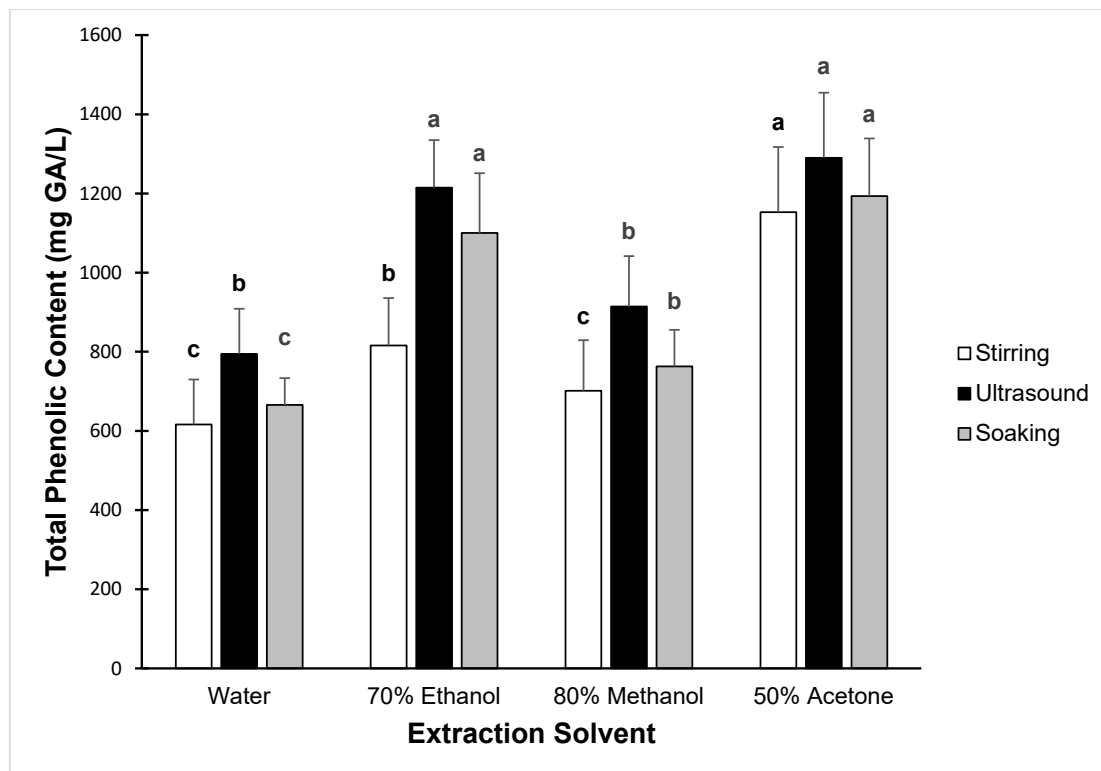


Figure 2. Total phenolic content (TPC) of olive leaf powder extracts using four different solvents (aqueous ethanol 70 vol%, aqueous methanol 80 vol%, aqueous acetone 50 vol%, and distilled water) across three extraction methods: stirring, soaking, and ultrasound-assisted extraction (UAE). TPC was determined using the Folin–Ciocalteu assay and is expressed as mg of gallic acid equivalents (GAE) per liter of extract. Data is presented as mean $\pm$ standard deviation ($n = 3$). Different lowercase letters above bars indicate statistically significant differences ($p < 0.05$) between solvents within the same extraction method, as determined by Bonferroni's post hoc test. No statistical comparison was conducted between different extraction methods due to differing conditions.

3.2.1. Stirring Extraction

Aqueous acetone again outperformed the other solvents, yielding 1152.70 ± 164.60 mg GAE/L (Table 2, Figure 2). Extraction in aqueous ethanol and aqueous methanol showed

intermediate values, while aqueous extracts presented the lowest TPC (616.19 ± 113.60 mg GAE/L), showing statistically significant differences ($p < 0.05$)

3.2.2. Soaking Extraction

Figure 2 graphically presents the results obtained following the soaking extraction method. The results show that soaking enhanced TPC for all solvents, while aqueous acetone and aqueous ethanol extraction performed better than the aqueous methanolic and aquatic extractions (Table 2), showing significant differences ($p < 0.05$).

3.2.3. Ultrasound-Assisted Extraction

As documented in Figure 2, ultrasound-assisted extraction maximized TPC for all solvents, especially using aqueous acetone (1289.95 ± 191.17 mg GAE/L) and aqueous ethanol (1214.76 ± 190.74 mg GAE/L) as solvents, while aqueous methanol and water were significantly ineffective ($p < 0.05$) (Table 2). The dominance of aqueous acetone and aqueous ethanol were indeed highlighted by statistical tests.

In summary, the results revealed that the aqueous acetone with intermediate polarity consistently yielded the highest TAC and TPC across all methods. Ultrasound-assisted extraction outperformed shaking and soaking, possibly due to enhanced cell wall disruption. Aqueous acetone and aqueous ethanol showed significantly superior performance ($p < 0.05$).

4. Discussion

The present study explored the antioxidant capacity and total phenolic content of olive leaf powder using different solvents and extraction techniques, aiming to identify optimal conditions for maximizing bioactive recovery from this agricultural by-product. Our findings align with previously published works showing that both solvent nature and extraction method significantly influence the recovery of bioactive compounds from olive leaves.

To better understand these differences in extraction performance, we selected solvents based on their reported efficiency in the literature regarding bioactive extraction from plant matrices, their relevance to food applications, and their potential to act as analytical tools. Specifically, water was chosen for its direct applicability in food matrices and established safety, whereas the organic solvents served an additional purpose. They acted as “model” solvents to quantify the full extraction potential of the olive leaf powder by providing an upper-bound benchmark for its antioxidant and phenolic content [12,13]. This is due to their high polarity and solvent strength that is known to yield greater amounts of bioactive compounds compared to aqueous extractions, thus providing a clearer image of the compound’s full recovery potential [14,15]. By comparing the organic solvents’ performance to the aquatic extraction method, we determined the efficiency of our extraction methods.

Across all methods, acetone (50% *v/v*) yielded the highest TAC and TPC values across most extraction methods. Specifically, it exhibited statistically significant differences ($p < 0.05$) compared to all other solvents in the stirring and soaking methods and outperformed distilled water and ethanol in ultrasound-assisted extraction. In the latter case, no significant difference was observed between acetone and methanol. In contrast, distilled water consistently demonstrated lower extraction efficiency, highlighting the potential trade-off the food industry faces between solvent safety and extraction efficiency. Aqueous ethanol (70% *v/v*) also yielded strong outcomes in both ultrasound and stirring conditions depending on the assay, offering a middle ground between safety and effectiveness, especially since its use is more widely permitted by regulatory bodies such as the EFSA and FDA for use in food applications [10,11]. This is provided that their residues are effectively removed from the final food product in order to avoid potential toxicity concerns, and this can be achieved using techniques such as rotary evaporation,

evaporation under reduced pressure, and lyophilization, all of which reduce residual solvent levels to food-grade safety standards [16–18].

These findings align with previous studies suggesting that water and pure organic solvents are less effective in ensuring the recovery potential of bioactive compounds than aqueous mixtures of the organic solvents, particularly in concentrations of 50% *v/v* or higher [19,20]. This enhanced efficiency is attributed to the intermediate polarity of these solvents due to the presence of both water and organic matter, which enables simultaneous solubilization of a broad spectrum of phenolic compounds, from highly to moderately polar. These mixtures also enable greater disruption of plant cell structures, thereby improving the release and recovery of bioactive compounds [21,22]. Specifically, Do et al. achieved the highest yield of antioxidant extraction in *L. aromatica* extracts using 50% aqueous acetone as a solvent. Their extracts also demonstrated the highest TPC when extracted by aqueous acetone and aqueous ethanol in various concentrations with a minimum of 50% [19]. Additionally, Van Ngo et al. also declared acetone 50% *v/v* as the optimal solvent for extracting phytochemical compounds, e.g., phenolics, showing that it achieved the highest antioxidant capacity following their testing of different organic solvents on *S. chinensis* sample extraction efficiency [20]. Similarly, ethanol-based aqueous solutions also demonstrated high extraction efficiency in these studies, aligning with our findings and highlighting ethanol as a promising alternative due to its comparable performance and well-established safety for use [19,23].

The extraction method itself proved equally as critical as the choice of solvent. Ultrasound-assisted extraction (UAE) yielded the highest TPC values across all solvents and outperformed other methods in TAC for most solvents. This finding supports the growing body of literature that recognizes ultrasound-assisted extraction alongside microwave-assisted extraction as the current state-of-the-art methods for extracting phenolic and antioxidant compounds from food matrices, since they consistently outperform conventional methods in terms of efficiency, time, and yield [24]. This method's high efficiency can be attributed to the enhanced cavitation effects from the ultrasound, leading to more effective cell wall disruption and mass transfer [25]. As a result, UAE is becoming increasingly recognized as a sustainable and energy-efficient extraction technology for phytochemicals, offering several advantages that make it highly attractive for industrial applications.

Interestingly, aqueous methanol (80%) and aqueous ethanol (70%) especially showed fluctuating performance depending on the method, suggesting that solvent–matrix interactions are not exclusively influenced by polarity but also by other factors, such as temperature, extraction time, or environmental conditions. For instance, aqueous ethanol demonstrated higher TAC when extracted using the stirring method compared to UAE, but it presented opposite results for its TPC. Those inconsistencies between assays can mainly be attributed to the fact that stirring and soaking are deemed as gentler, longer processes that employ different kinetics, which, at times, can be more effective at recovering a broader range of antioxidants besides phenolics, therefore increasing TAC. In contrast, UAE is a more aggressive technique that is highly effective in quickly disrupting cell walls and extracting phenolic compounds but may simultaneously cause degradation of other less stable antioxidants compounds. Cacique et al. reported similar findings, demonstrating that extraction without stirring can yield higher phenolic concentrations than agitated systems. This suggests that aggressive mechanical action does not always enhance phenolic extraction and may be less suitable for recovering non-phenolic antioxidants [26].

In conclusion, our findings provide insight into the possible incorporation of olive leaf powder in functional food applications by demonstrating its value as a potential ingredient rich in bioactives. By employing practical extraction strategies and widely used solvent systems, our study contributes to the broader effort of valorizing agricultural by-products

while promoting circular economy principles. While organic solvents yielded higher overall antioxidant and phenolic recovery, ethanol and water remain the safest choices for industrial application, assuming minimal residual solvent levels. Although aqueous acetone and aqueous methanol demonstrated superior extraction profiles, their use in food matrices would require strict adherence to purification protocols to ensure compliance with regulatory limits on residual solvents, such as those outlined in the ICH Q3C and EFSA guidelines [18]. However, they serve as reference points for determining the maximum extraction potential under ideal conditions. Methodologically, the optimal approach depends on the target compounds. Specifically, stirring favored total antioxidant recovery, while ultrasound-assisted extraction was more efficient for phenolics. The current lack of standardized extraction and analytical protocols across the literature, however, complicates comparisons between studies and highlights the need for standardized methodologies.

It should be underlined that a very important limitation of the study is the fact that we did not use rigorous chromatographic methods or techniques such as GC-MS, LC-MS, or HPLC to establish concentrations of specific metabolites of interest. Specifically, a serious limitation of this study is the absence of compound identification, such as HPLC profiling of individual phenolics (e.g., oleuropein, hydroxytyrosol), which could offer a deeper mechanistic understanding. Future studies should integrate chromatographic analysis and assess the bioavailability and stability of these compounds in food matrices.

Author Contributions: Conceptualization, O.P., C.D. and A.E.K.; data curation, O.P., P.P. and C.D.; formal analysis, P.P. and C.D.; funding acquisition, A.E.K.; investigation, K.A., P.M., S.K.T. and P.P.; methodology, K.A., O.P., P.M., S.K.T., P.P. and C.D.; project administration, A.E.K.; resources, K.A., P.M., S.K.T. and A.E.K.; software, K.A., P.M., S.K.T. and P.P.; supervision, A.E.K.; validation, O.P. and P.P.; visualization, O.P., C.D. and A.E.K.; writing—original draft, K.A. and O.P.; writing—review and editing, A.E.K. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Rural Development Program (RDP) 2014–2020 713 within the framework of Action 2 of Sub-measure 16.1–16.2 “Establishment and operation 714 of Operational Groups of the European Innovation Partnership for the productivity and 715 sustainability of agriculture” (code: M16SYN2-00375).

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: The authors sincerely thank all colleagues and laboratory staff who contributed to the successful completion of this study.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AU	Absorbance Units
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
FRAP	Ferric Reducing Antioxidant Power
HPLC	High-Performance Liquid Chromatography
ICH	International Council for Harmonisation
TAC	Total Antioxidant Capacity
TPC	Total Phenolic Content
UAE	Ultrasound-Assisted Extraction

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